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**Generation of Fluorescent Protein-single Chain
Antibody Fusion Products as Bio-detection and
Identification Reagents (*Final Report*)**

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FINAL REPORT FOR PROJECT ENTITLED:

Generation of Fluorescent Protein-single Chain Antibody Fusion Products as Bio-detection and Identification Reagents

Contract No. W7702-6-R600/001/EDM

Buyer ID.edm610

Regional File No. EDM-6-00625

(Period from July 31, 1996 to March 31, 1997)

**Submitted to: Science and Professional Services
Western Region, Public Works and
Government Services Canada**

and

**Medical Countermeasures Section,
Defence Research Establishment Suffield**

**Submitted by: S. Jager, RTM
J.K. Pon, RTM
M. Mah, QAO
D. Mah, Projects Director**

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**Canada West
Biosciences**

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Executive Summary

We have genetically fused an the gene encoding recombinant GFPuv to the 3' end of anti-BoNT/A D12 scFv antibody gene in expression vector pRSD12-8. This scFv-GFP fusion vector which is under the control of the T7 promoter expresses the D12-GFPuv fusion protein (60.6kDa) as non-fluorescent inclusion bodies. Inclusion bodies can be denature in 8 M urea and purified on IMAC (immobilized metal affinity chromatography) columns, but attempts at renaturation failed to regenerate a fluorescent fusion product. We hypothesize that the loss of fluorescence is due the improper folding which occurs as a result of high-level expression and/or misguiding of protein folding by the preceding scFv polypeptide during protein synthesis. Work was initiated to construct an GFP-scFv fusion expression vector under the control of the lacZ promoter to test these hypothesis.

This contract delivers a plasmid DNA and/or bacterial glycerol stocks of T7 promoter-based scFv-GFP fusion expression vectors (pRSD12guv-1 or pRSD12guv-2) and intermediate plasmid constructs for a GFP-scFv fusion expression vector (pGuvD12). In addition, seventeen (17) anti-ovalbumin mouse monoclonal hybridoma cell lines (derived from 5 primary clones) are delivered along with one anti-ovalbumin scFv clone (p12H6H8F3) in phage-display vector pCantab5E.

The nature of genetically cloned antibody genes facilitates work to improve the usefulness of recombinant scFv-GFP fusion antibodies as fluorescence-based immunodetection reagents. Two aspects of antibody function, 1) antigen-combining properties and 2) recombinant GFPuv fluorescence, can be potentially improved by mutagenesis of the scFv protein domain and GFPuv domain respectively. The bacterial genetic clones obtained in this work can be used in mutagenesis experiments to improve antigen combining properties and fluorescence of scFv-GFP fusion antibodies.

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Abbreviations:

ABTS	-2'2'-Azino-Bis (3-Ethylbenzthiazoline-6-Sulphonic Acid) Diammonium
Ab(s)	- antibody(ies)
AFM	- atomic force microscopy
BIA	- biomolecular interaction assay
BFP	- blue fluorescent protein
BoNT/A	- botulinum neurotoxin serotype A
bp	- base pairs
CDR	- complementarity determining region
CE	- capillary electrophoresis
DEPC	- diethyl pyrocarbonate
D12-GFP	- anti-BoNT/A scFv antibody fused to the N-terminus of GFP.
EK	- enterokinase peptide cleavage site
ELISA	- enzyme linked immunosorbent assay
Fab	- antibody fragment antigen binding--comprised of Fd and Lc fragment.
FACS	- fluorescent activated cell sorter
Fc	- antibody fragment crystallization -- contains Hc -CH2 and CH3 domains
Fd	- antibody fragment diverse -- single-chain VH joined to CH1
Fv	- antibody fragment variable -- VH/VL
GFP	- green fluorescent protein
GFP-D12	- GFP fused to the N-terminus of D12 scFv antibody
GFP-scFv	- GFP fused to the N-terminus of scFv antibody
GFPuv	- GFP mutant with max. fluorescence with uv irradiation
6His	- six histidine peptide domain.
Ig	- immunoglobulin
IMAC	- immobilized metal affinity chromatography
IMFET	- immunofield effect transistor
kb	- kilobases
kDa	- kilodaltons
Lc	- light chain
LIF	- laser induced fluorescence
LMPA	- low melting point agarose
LP	- liquid-phase
mAb	- monoclonal antibody
pAb	- polyclonal antibody
PEG	- polyethylene glycol
PCR	- polymerase chain reaction
PMSF	- phenylmethyl sulphonyl fluoride
PS-beads	- polystyrene beads
rAb(s)	- recombinant antibody(ies)
rGFP	- recombinant GFP
rM13	- recombinant M13
ScAb	- single-chain antibody
scFv	- single-chain Fv
scFv-GFP	- scFv antibody fused to the N-terminus of GFP
SP	- solid-phase
VL	- variable light domain/region
VH	- variable heavy domain/region
YFP	- yellow fluorescent protein

Review of Intrinsically Fluorescent Proteins

Overview

Bioluminescence, the production of visible light by living organisms have provided biologists with tools that increase the understanding of many biological processes. This has been possible by isolation of some of these naturally occurring proteins and introducing them into cells as fluorescent tags ^{2,3}.

Fluorescence occurs when a fluorescent molecule or fluorophore absorbs light from an external or excitation source and then emits the energy subsequently. The emitted light is of a lower energy (longer wavelength) than the absorbed light due to some loss of the excitation energy through vibrations at the molecular level, collisions, movements and other dynamic processes that occur during the nanoseconds excited state of the fluorophore. The resulting shift between excitation and emission (the Stokes Shift) enables differentiation between background light and fluorophore specific emission. Depending on the nature of the fluorescent measurement, different information can be ascertained about the fluorophores themselves or about the molecules tagged. Key spectral parameters used in fluorescence detection are 1) the excitation and emission wavelengths at which maximum excitation and emission efficiency occur and 2) the extinction coefficient which is indicative of the efficiency of the ability to absorb excitation energy, at a particular wavelength ⁵³.

Naturally occurring photoproteins

Many different organisms (firefly, bacteria, fungi, jellyfish etc.) are bioluminescent (i.e. have ability to emit light). The molecular mechanisms by which proteins of bioluminescent organisms generate light can range from enzymatic oxidation of a substrate (eg. firefly luciferase oxidation of luciferin - light-bearing molecule), and noncovalent association/interaction with a chemical chromophore (bacteria) to harbouring intrinsic peptide chromophores (jellyfish GFP) [Table 1] ^{2,6}.

The colors of bioluminescence among different organisms differ from green to red to yellow. Emission spectra for many members of luminous species have been reported. The color appears to be associated to some extent by habitat with blue emissions among those in deep waters, green (490-520nm) by those in the coastal marine areas and 550-580nm for terrestrial and fresh water species ⁶.

Table 1. Summary of Proteins Involved in Bioluminescence ⁶

<u>Name</u>	<u>Source</u>	<u>Requirements</u>
Firefly Luciferase	Firefly	luciferin substrate, (benzo)thiazole ATP, divalent cations.
Aequorin Luciferase	Jellyfish	luciferin substrate, divalent cations. (Ca ²⁺)
Renilla Luciferase	Sea Pansy	luciferin substrate, O ₂ .
Obelin Luciferase	hydra	luciferin substrate, divalent cations (Ca ²⁺).
Vibrio Luciferase	bacteria	luciferin substrate, FMNH ₂ , RCHO
Gonyaulax Luciferase	dinoflagellates	luciferin substrate Tetrapyrrole; H ⁺
Diplocardia Luciferase	annelid	luciferin substrate; N-isovaleryl-3-amino propanal, H ₂ O ₂
Latia Luciferase	molluscs	luciferin substrate; enol formate; terpen or aromatic aldehyde
Vergula Luciferase	crustacea	luciferin substrate; imidazopyranzine nucleus
YFP	bacteria	chemical chromophore
GFP/BFP	Jelly fish	Intrinsically fluorescent-excited by blue light

Luciferases

This class of bioluminescent photoproteins which requires substrate (luciferin) and often cofactors to produce light, is by far the largest known class of photoprotein. They naturally occur in bioluminescent species of insects, coelenterates and bacteria ⁶. Biochemically, all known Lase (Luciferase) are oxygenases that use molecular oxygen to oxidize a substrate, a luciferin, to ultimately form a product molecule in an excited state which emits light ⁶. The bioluminescent systems in the diverse variety of organisms are chemically different except for the involvement of O₂ ⁶. Oxygen has an important role in the ultimate formation of a product molecule in the excited state through the process of chemical oxidation (Luciferase or generically known as Lase⁶).

The color of light produced by the different luciferase is dependent on the structure of the luciferin substrates. The structure of luciferin for insect species was determined to be a benzothiazole, which in different systems will result in different colors. McCapra et al. proposed that the color is determined by the relative orientation of the two luciferin rings and peak emission can fall anywhere ⁵⁵ from orange, yellow to green. The substrates or luciferins, fall into many different chemical classes. Tetrapyrroles in Dinoflagellates, coelenterazines in Cnidarians, H_2O_2 in Annelids and benzothiazoles in insects indicate the substrates also have different evolutionary origins ⁶.

Firefly luciferase (Luc) is one of the first photoproteins studied has an absolute requirement for divalent cations and ATP ⁷³.

Aequorin (21.4 kDa) is a luciferase-type photoprotein that its final product emits a high energy blue light which GFP transduces into a lower energy green light ¹⁵. Aequorin (from *Aequorea victoria*) consists of a Ca^{2+} binding apoprotein (apoaequorin), coelenterazine (an organic substrate) and molecular oxygen. In vitro, the binding of Ca^{2+} to aequorin triggers an intramolecular reaction in which coelenterazine is oxidized to coelenteramide producing a protein which appears fluorescent blue, CO_2 and light at 470nm ⁷.

Obelin is a bioluminescent photoprotein isolated from the marine hydroid polyp, *Obelia longissima* ¹⁴. whose luminescent action is initiated by binding to Ca^{2+} . Obelin consists of a single polypeptide chain ($M=22.2$ kDa) and complexes with apoobelin and coelenterazine (an imidazolopyrazine compound) through molecular oxygen. Its luminescent reaction occurs as a result of an intramolecular reaction of coelenterazine oxidation yielding coelenteramide, CO_2 and 470nm light. Cloning and sequence analysis of cDNA for apoobelin has shown that obelin is made up of 195 amino acid residues ⁴⁷.

Protein fusion to the N-terminus of obelin is possible without the loss of luminescent activity. The expression of an apoobelin fusion protein with the ZZ-domain of *S. Aureus* protein A has produced a universal marker in sandwich format immunoassay efficient at binding various immunoglobulins ¹⁸. Obelin mRNA can be used to monitor the efficiency of a translation system, assessing the accumulation of biologically active protein or used as a template to evaluate the kinetics of accumulation of the target protein in a cell-free system by luminescence avoiding the use of labelled amino acid ³⁴.

RL or Renilla luciferase (36 Kdal) is isolated from the sea pansy (*Renilla reniformis*). It is immediately functional following translation, doesn't require divalent cations or ATP. RL in the presence of O_2 alone reacts with its natural substrate coelenterazine to yield oxy-luciferin, CO_2 and

blue light 71, 72.

Applications using luciferases

Vectors carrying genes encoding luciferase from *Vibrio harveyi*, were used in studies of gene expression in Gram positive *Bacillus subtilis* ²⁶. These vectors contain the bacterial luciferase genes from *V. harveyi* which encode a heterodimer enzyme capable of producing light quanta in response to molecular oxygen, reduced FMN(flavin mononucleotide) and a long -chain aldehyde ²⁹. Luciferase genes from *V. fischeri* were cloned in an *E.Coli-Bacillus* vector to use the genes of *V. fischeri* for studies of the *B. megaterium* sporulation cycle. Sporulation-specific sequences were found on the basis of light emission at different sporulation stages. The studies concluded that the in vivo light measurements from non-disrupted living cells give a good indication of events inside the cell at the protein synthesis level. *E.Coli* was shown to produce three hundred times more light than the corresponding *B.subtilis* clones produce. The difference can be explained by consensus gram-negative like ribosome binding sites in front of lux A and B of *V. harveyi*. *E.Coli* - like ribosome binding sites have been shown to be ignored in *B.subtilis* ²⁶.

A chimeric protein that consisted of Protein A and luciferase from the sea-firefly *Vargula hilgendorffii* has been constructed and used as a tool for bioluminescent immunoassays ⁶³. The primary structure of the luciferase consists of 555 amino acids with two potential glycosylation sites. The light emitting reaction is very simple consisting of the oxidation of luciferin by molecular oxygen in the presence of *Vargula* luciferase.

A genetic fusion between the streptavidin of *Streptomyces avidinii* and the luciferase of *Pyrophorus plagiophthalmus*, a Jamaican click beetle, has constructed. The fusion protein was produced in an insect sf9 cell line using the baculovirus expression vector technology. The recombinant baculovirus can be used to produce a functional fusion protein containing the entire coding sequences of luciferase and streptavidin in lepidopteran insect cells ⁶⁴.

YFP (Yellow Fluorescent Protein)

Functionally, YFP is the most similar to GFP in that it functions as a secondary fluorescent protein to a bacterial luciferase (Lux). A yellow-light emitting bacterium discovered by Ruby in 1977 peaks was found to emit light at both 540nm (peak) but also 490 nm. Lux activity on substrate alone produced light emitted at 490 nm. The energy generated from Lux was found to be transferred to the second protein, YFP to reaction produced the peak light emission at 540 nm. YFP does not have a peptide fluorophore as does GFP; its fluorophore is a noncovalently associated FMN or riboflavin ^{6,21}. YFP from *Vibrio fischeri* strain Y1 was found to work well in generating

bioluminescence with a luciferase from *Photobacterium leiognathi* ²¹. In *Photobacterium leiognathi*, the equivalent YFP equivalent is a twenty-one kDa protein called "lumazine protein" and it's noncovalently associated fluorophore is 6,7-dimethyl-8-ribityllumazine ^{21,62}.

YFP fluorescence chemistry, characteristics and functions

The bacterial luciferase reaction involves the binding of reduced flavin to Lux and a reaction with O₂ to form a peroxide which then reacts with an aldehyde to give a blue green light, oxidized flavin and acid. A transient luciferase bound intermediate, 4a-hydroxyflavin, is the emitter. Its fluorescence is found transiently in the reaction product before the hydroxyflavin breaks down with loss of water to give FMN ⁶. Light emitted from the Lux reaction stimulates YFP to fluoresce with a light emission of 540 nm. Effects are greater at lower temperature both in vivo and in vitro. At 4°C YFP not only changes the color of the emission in vitro, it increases the rate of reaction by tenfold ⁶. YFP does not bind to luciferase itself so it must interact with one of the intermediates prior to the formation of the excited state.

Applications

Uses include facilitation of multicolor imaging of differential gene expression, protein localization and cell fate. Fusions between mutants of different colors may be useful substrates for in situ assays of proteases. In energy transfer rate studies, there was a significant advantage in using yellow fluorescent protein in that its fluorescence spectrum is well separated from the donor fluorescence ²¹.

GFP (Green Fluorescent Protein)

Green Fluorescent Protein (27 kDa), predominantly from the jelly fish species *Aequorea victoria* and also from the sea pansy, *Renilla reniformis* ^{3,5}, has been extensively used in the microscopy studies of intracellular expression in eukaryotic cells. The proteins from these organisms are not identical, but they are thought to have the same chromophore which is derived from the primary amino acid sequence of GFP. However the strong tendency of *Renilla reniformis* to dimerize might complicate its use as a fusion tag ³. In the natural system, fluorescence of GFP is generated by sequential activation of two photoproteins; aequorin activity produces a blue light which excites the green fluorescent protein to fluoresce ^{8,15}. In vitro when this protein is illuminated with ultraviolet light (365 nm) or blue light (395 nm), GFP gives off a green fluorescent light (508 nm) without additional co-factors.

Genetic Engineering of GFP

The GFP of *A. victoria* has cloned into *E. Coli*. The recombinant, rGFP, was isolated

and purified using two chromatographic steps: size exclusion chromatography and ion-exchange HPLC. The hydrophobic nature of the protein, plus the surface characteristics of the HPLC size column and the high initial salt concentration allows the rGFP to stick to the size column and be eluted by reducing the salt concentration ⁵⁸.

Recombinant *Aequorea* GFP-6His fusion product expressed in *E.Coli* yielded a fusion product with fluorescence excitation and emission identical to the native GFP. The expression vector produced a fused protein ~31kDa in *E.Coli*. The protein had a 34 amino acid polypeptide having six histidine residues in tandem at the N-terminus plus three amino acid residues originating from the PCR primer at the fusion junction and the GFP. The expressed protein was absorbed by a Ni-loaded gel and formed a bright green fluorescent band which was eluted with imidazole ⁷.

Many mutant of GFP have been made which have change the color and intensity of fluorescence. Such mutants have been useful for the studies examining simultaneous events where GFP variants fluoresce at different emission colors. ¹ The emission color of GFP is shifted by substituting histidine or tryptophan for the tyrosine in the chromophore. Substitutions, mainly in residues 145-163 have improved the brightness of the blue-shifted GFP mutants with histidine and tryptophan in place of tyrosine 66. Excitation wavelengths and intensities, emission wavelengths and intensities and protein stability characteristics are GFP properties that have been altered by various mutagenic manipulations ^{1,19}. Mutants of GFP have been constructed which emit red fluorescent light and/or emit a stronger fluorescent signal.

Cramer, A. et al ¹¹ constructed a synthetic GFP gene with improved codon usage by performing recursive cycles of DNA shuffling followed by screening for the brightest *E.Coli* colonies. After three cycles of DNA shuffling, a mutant was obtained with enhanced fluorescence and faster growth rate, without altering the expression level and emission characteristics. Whereas in *E.Coli* most of the wildtype GFP ends up in inclusion bodies, unable to activate its chromophore, most of their mutant protein was soluble and active. Three amino acids appear to guide the mutant protein into the native folding pathway rather than towards aggregation ¹¹. But the aforementioned work comprising the mutant were difficult to explain based on the molecular structure study by Fan, Y. et al ¹⁹.

Mutations in the range of positions 65-67 adjacent to the chromophore have been explored ²⁰. Some of these have been found to cause significant wavelength shifts but most cause a loss in fluorescence intensity ¹⁹.

Two GFP mutants with excitation maxima around 490nm have been created. One red-shifted mutant was created by replacing three amino acids ²⁰, the other by a single amino acid

substitution of serine-65 to threonine. This shift allows detection by common flow cytometers containing an Argon ion laser and were shown to fluoresce brighter than wt.GFP. These mutants were expressed in mammalian cells ⁵⁷ and extended its applications to include monitoring gene transfer and expression ²⁵ in gene therapy protocols, monitoring gene expression during development and disease states and screening drugs which modulate the regulatory elements of gene expression.

GFP was expressed from *A.victoria* in the secretory pathway of HeLa cells by fusing it to the C-terminus of a secretory protein, chromogranin B, and offered the possibility to analyze transport in the secretory pathway of living cells ^{32, 33}.

DNA shuffling is an invitro PCR based recombination technique. Genes with similar nucleotide sequences are pooled and randomly nicked in a controlled digestion reaction. Without added primers, the fragments denature to single strand fragments which are allowed to randomly anneal often to new partners. These prime each other in polymerase catalyzed extension reactions that fill in the overlapping regions. Many cycles later, the full length genes are regenerated simultaneously introducing random point mutations. The recombinant products are PCR amplified with conventional primers and subcloned into an expression vector for screening and selection. With DNA shuffling, colonies were selected that contained brighter variants of GFP with the result of one having forty-two times the intensity of the wild type ⁴³.

GFP Fluorescence Chemistry, Characteristics and Functions

Using multi -wavelength anomalous dispersion phasing methods, GFP was found to be in the shape of a cylinder, made of eleven strands of beta sheet on the outside with an alpha helix inside and short helical segments on the ends of the cylinder. The outer beta and the inner alpha structure represents a new protein fold called the beta-can. A dimer in the crystal is formed by two closely packed protomers. The chromophore is composed of modified amino acid residues within the polypeptide ¹⁶. Highly protected, the chromophore is located on the central helix almost at the center of the cylinder. The pocket containing it has a number of charged residues and apolar and polar acid side chains in close proximity to it ¹⁹. The fluorophore (chromophore) are protected inside the cylinders and the former are formed by aromatic systems made up of Tyr-66 with cyclization of the neighbouring glycine and serine residues (Figure 1). Structure studies suggest specific side chains close to the chromophore could be modified to effect spectral properties and dimer contacts may allow mutagenic control of the state of assembly of the protein. The fluorophore is near the geometric center of the molecule to protect it from collisional quenching by oxygen. Two resonant forms of the fluorophore exist: one with a partial negative charge on the benzyl oxygen of Tyr-66, the other with the charge on the carbonyl oxygen of the imidazolidone

ring. Basic residues appear to form hydrogen bonds with each of these oxygen atoms which help to stabilize and delocalize the charge on the fluorophore ¹⁹.

A key feature of the versatility of GFP is its capacity to undergo heterocyclic chromophore formation by cyclization of a tripeptide present in its primary sequence. This was demonstrated by GFP deletion variant studies showing lack of fluorescent activity when there was no evidence of heterocyclic ring structure formation ³¹. Shifts in the emission color of GFP have been performed by the replacement of histidine or tryptophan in the chromophore with tyrosine and additional amino acid substitutions in residues 145-163 ¹.

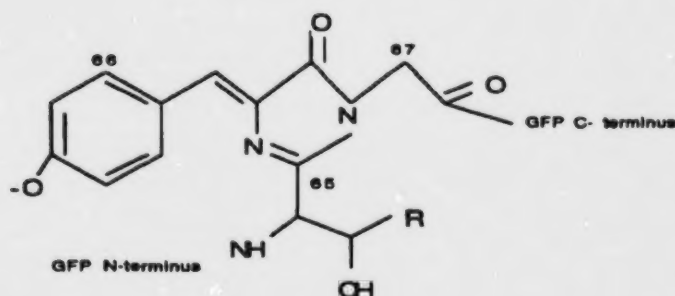


Figure 1. The GFP fluorophore. Blue light stimulates the excitation of the cyclized wt GFP fluorophore formed by serine -65, tyrosine-66 and glycine 67, emitting a green fluorescence.

GFP, which acts as an energy transfer acceptor, emits light when energy is transferred from a luciferase-oxyluciferin complex or a Ca^{2+} activated photoprotein, aequorin in the form a blue light. ⁵ GFP is naturally fluorescent when excited by light from one wavelength, and emits light of another wavelength. This characteristic is called fluorescence resonance energy transfer. This quantum mechanical event occurs when an excited fluorescent group is located near a light-absorbing group. If the distance and orientation are optimal and the energy of the excited fluorophore matches the energy needed to excite the absorber, then the energy is transferred. GFP absorbs blue light, maximally at a wavelength of 395 nm and emits green light at 509 nm.

Recent mutagenesis data indicate changes in the fluorescence excitation spectrum as a function of pH. At high pH or after certain mutations are introduced near the GFP fluorophore, Tyr 66 is postulated to be in the phenolate form and in the hydroxy form at low pH. While both forms absorb light, only the phenolate form fluoresces ¹⁵.

GFP fluorescence is not species specific. it does not form multimers that might interfere with function of protein to which it is fused. In situations where there is no fluorescence, it is not clear whether the defect lies in the stability of the expressed product or in the formation of the

chromophore².

The successful use of GFP in protein fusions has a structural basis in the tight compact protein fold, with no carboxyl or amino terminus involvement of this protein. The ends are free for His-tagging or fusion to other proteins ^{7,15}.

Use of GFP in Studying Biological Process.

Although the properties of the protein have been studied for a long time, it was not until the protein had been cloned and expressed that its wide array of applications have been exposed ⁸. These applications include using GFP as an in-vivo reporter for monitoring processes in organisms (eg. zebra fish embryos, ¹³) or cells ¹² (eg Hela cells ¹⁷), as a fusion tag GFP ⁹, to follow, localize and study the dynamics of the areas to where the proteins are targeted, ie. migration studies on a cellular level ⁵⁶ involving *C. elegans* or at a particle level ⁴⁵ involving *Drosophila* oogenesis.

Experiments with *C. elegans* established that GFP is useful as a marker for gene expression ². A method to selectively produce transgenic mice using GFP as a marker has been used ⁴⁴. Before, the low efficiency of transgenic animal production by microinjection had been a serious problem for the production of transgenic livestock. But since GFP expression was observed in bovine blastocysts, using GFP as a fusion tag during mammalian development shows its use for mammalian cell-lineage analysis and thus contribute to the efficient production of bovine transgenic livestock as well.

Two bacterial strains were modified for use in bacterial transport experiments. The Tn5GFP transposon incorporates the gene(GFP) encoding green fluorescent protein (GFP) and was used to mutagenize the Gram negative bacteria and became a valuable tool for labelling bacteria ¹⁰.

GFP was used as an in vivo reporter protein, when a fusion protein was made between yeast hydroxymethylglutaryl-CoA reductase and GFP ²². The fusion protein displayed localization and regulated degradation consistent with the native reductase protein and showed its usefulness in studying membrane protein localization and protein degradation in vivo.

Its use as a fluorescent tag or microtubule-associated protein chimera, for cytoskeletal studies was praised for its stability, high fluorescence, low phototoxicity and photobleaching ³⁰.

GFP can act as a vital dye upon absorption of blue light. When the GFP gene is expressed in bacteria, flies, nematodes, e.t.c. green fluorescence can be directly observed in the living organisms. cDNA-encoding GFP was inserted into an expression vector containing rat myosin light chain enhancer to evaluate its ability as a muscle specific marker in zebrafish ⁴².

A genetic fusion between GFP and that of Ob-tobamovirus movement protein (constructed by placing the GFP gene in frame with the Ob mp gene) resulted in the expression of a fluorescent fusion protein that was fully biologically active in mediating the cell-cell spread of the Ob-virus ⁴⁰. This tagged protein was expressed and found to be associated with several subcellular compartments and structures ie. trans-wall structures, plasmodesmata and filament structures at all stages of infection. For simply tracking the spread of the virus, expression of GFP as a free protein using the promoter for the coat protein gene is a better strategy since the level of this expression is one-two times greater.

GFP and Aequorin have been molecularly engineered and produced in mammalian cells to serve as specific reporters of subcellular microenvironments. Aequorin was used to target three intracellular locations: cytosol, nucleus and mitochondria. Being Ca^{2+} sensitive, aequorin allowed the direct measurement $[\text{Ca}^{2+}]$ within these compartments. Whereas, the GFP provided specific fluorescent in vivo for identifying various subcellular structures ⁴¹.

Green fluorescent protein is a potentially powerful fusion tag for protein localization and translocation studies in intact cells. An ideal fusion tag would neither bind to itself nor to other cellular components within the cell. This can be done by measuring the diffusion coefficient of GFP, to compare its value to a freely diffusing molecule. Thus studies were undertaken to monitor the spatial dynamics of GFP proteins in intact cells by enhancing the blue excited fluorescence of GFP using a spatially focused ultra-violet laser pulse ²⁸. GFP fusion proteins were efficiently expressed by micro-electroporation of invitro synthesized mRNA into adherent mammalian cells to test its efficiency. Mutant GFP's were generated using DNA shuffling ^{11,43} to produce an ideal fusion tag ³. Wild-type GFP gave a poor diffusion coefficient in comparison, possibly because wt.-GFP may have a hydrophobic surfaces that causes self-aggregation ¹¹.

Two GFP expression vectors have been constructed for 1) enteric pathogens, *Salmonella typhimurium* and *Yersinia pseudotuberculosis* and 2) for *Mycobacterium Marinum*. Both expression vectors were found to be stable and to direct high levels of GFP ³⁹ in hosts. In these tests, the visualization of GFP expressing bacteria in infected animal tissue suggests the use of GFP -producing bacteria as probes to determine host cell interactions in infected hosts and identify subsets in complex environments such as the gut. The use of GFP as a biological tracer will allow monitoring of colonization, multiplication, persistence and transmission of pathogenic bacteria in hosts and even host populations.

The combined use of GFP and EGFP mutants permits the simultaneous analysis (measurement of two emission spectra) of two promoter elements, the localization of two

different proteins, the monitoring of different cell lineages in a single organism and the analysis of interactions between two distinct protein fusions ⁵².

An experiment expressed a chimeric gene encoding a fusion between the *Aequorea victoria* green fluorescent protein and the amino terminus of the Potato virus X coat protein which was assembled into virions ³⁸. Its presence in infected cells were similar to those seen in wild type infection. This detection of virus particles in infected tissue demonstrates the potential of fusions between the green fluorescent protein and virus coat protein for the non-invasive study of virus multiplication and spread.

Use of GFP in other detection applications.

GFP fluorescence on cells can be readily be detected by fluorescence-activated cell sorter (FACS) analysis. FACS studies show that it can be used as a fluorescent tag in a random cDNA bacterial library to sort expressing from non-expressing cell populations ^{2,65}. Measurement of biomolecular fluorescence of biological particles by FACS flow cytometry coupled with non-specific techniques such as particle counting can greatly reduce incidences of false alarms ⁴⁹. Possibly using BWA-specific fluorescent-antibody conjugates to immunostain collected particles, the signal to noise ratio can be enhanced to positively identify the sample.

GFP fusion products of viral proteins have been used as markers which allow virus particles to be quantitated spectrophotometrically using fluorescence instead of using plaque titration ⁶⁶.

GFP-calmodulin fusion product has been uses as a "western blot" reagent to stain calmodulin-binding proteins in cellular protein samples blotted onto membranes ⁶⁷.

Two isoforms of GFP joined by a calmodulin sequence was found to undergo conformational changes to quench emitted light upon binding to calcium ^{68, 69}. The work establishes the principle that the appearance GFP fluorescence can be coupled to conformational changes upon binding of a GFP fusion protein to its ligand.

Technical Considerations and Detection

- a) It is essential to avoid high Xenon lamps that cause rapid destruction of GFP.
- b) The stability of the protein varies with organism being studied with high stability demonstrated in *Drosophila* and zebrafish systems ⁵.
- c) Chromophore formation is dependent on the oxidation of Tyr 66. Formation of the chromophore is temperature sensitive with the amount of fluorescently active GFP decreasing at temperatures greater than 30°C. *E. coli* show strong fluorescence at 24 or 30 °C while mammalian cells show more fluorescent at 30°C ⁵.

- d) Reducing agents can convert protein to non-fluorescence but it can be recovered after exposure to atmospheric O₂.
- e) Fluorescence is retained in mild denaturants (1% SDS), pH range of 7-11.
- f) Cells producing high levels of GFP appear to be susceptible to illumination, possibly due to free radicals generated by the GFP fluorophore ⁹.
- g) GFP is resistant to denaturation requiring 6M guanidine hydrochloride at 90 °C or pH below 4 or above 12. Partial or near total renaturation occurs following reversal of denaturing conditions by dialysis or neutralization ⁴⁶.
- h) Renaturation with 6M guanidine hydrochloride at elevated temperatures (2min t 92°C for 2min.) or acid treatment (pH 2.0 for 5 minutes) could be partially renatured ^{46, 48}.
- i) A variability in the intensity of GFP fluorescence due to the slow formation of the chromophore and the requirement of molecular oxygen.
- j) Formation of the GFP chromophore appears to be optimized at a lower temperature around 30°C.
- k) Photobleaching may occur if there is extended excitation at the 395nm peak for wt GFP. Optimal excitation wavelengths for wt GFP is 470nm and 360-400nm for GFPuv.
- l) When detecting fluorescence against a background, fluorescence generated by a medium containing riboflavin strongly reduces contrast ⁹.

BFP (Blue Fluorescent Protein)

BFP is a genetic mutant of GFP which was found to exhibit very different fluorescent properties (Table 2). Hein and Tsien isolated this mutant GFP with shifted excitation and emission spectra. This BFP mutant, also called Y66H, when excited in the UV, with the excitation peak at 380nm, emits blue light with an emission peak at ~450nm ⁵⁴.

Genetic Engineering of BFP

Considerable work has been done to improve the fluorescent properties of BFP. The simplest way to shift the emission color of GFP is to substitute histidine or tryptophan for the tyrosine in the chromophore, but these blue shifted point mutations are only dimly fluorescent. Additional substitutions, mainly residues 145-163 have improved the brightness of the blue-shifted GFP mutants with histidine and tryptophan in place of tyrosine 66 ¹. Recently however, mutants for GFP (sg25) and BFP (sg50) were identified that gave 100X's and 63X's increased fluorescence intensity respectively ⁷¹.

Table 2. GFP and BFP Mutants

Protein	Amino Acid Positions						Fluorescence		
	64	65	66	163	167	238	Ex	Em	RF
wt GFP	F	S	Y	V	I	K	398	509	1
sg25	L	C					474	509	100
BFP			H				383	447	1
sg50	L		H	A			386	450	63

Notes: Ex is maximum excitation wavelength (nm). Em is maximum emission wavelength (nm).
RF is relative fluorescence.

Summary

Of the three major groups of photoproteins reviewed above, GFP/BFP are the simplest potential genetic fusion partners that can be used to generate fluorescent or bioluminescent antibody reagents. Unlike luciferases, they do not require substrate or cofactors in order to produce light. Unlike YFP which has a noncovalently associated chemical fluorophore, GFP/BFP have an intrinsic covalent peptide-based fluorophore which is an integral part of the GFP amino acid sequence. The genetic fusion of GFP and/or GFP mutants with scFv antibodies may potentially provide fluorescence-based immunodetectors with useful assay reagents.

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Review of Recombinant Antibodies as Immunodiagnostic Reagents [ScAb and ScAb-fusion Products].

Animal antisera and monoclonal antibodies provide a very important source of antibody for the immunodetection of biological antigens (e.g. toxins) and biological agents (e.g. viruses, bacterial spores and bacteria). Single-chain antibody (scAb) products developed from advances in recombinant antibody technology (1-3) provides an alternative source of antibody which is amenable to genetic manipulation to obtain advantageous properties (4,5,6,7,8). Many scAb have been developed for use in treatment of diseases and medical therapy (4,5). Immunotoxins for cancer treatment have been generated using scFv antibodies, and diphtheria toxin and pseudomonas exotoxin A (9, 10). As with conventional antibodies, recombinant scFv or Fab antibodies can be readily labelled with radioisotopes (^{125} I) and use for radioimaging of tumors in cancer medicine (10). These same isotope-labelled recombinant antibody reagents are amenable for use in other radioimmunodetection assays. However, scAb or scFv (single chain fragment variable), and other recombinant antibody proteins (e.g. Fab) have also been developed for unique uses in the immunodetection of certain antigens.

ScAb-Alkaline Phosphatase Fusion Product

Anti- 6His scFv (3D5) has recently been genetically fused to the N-terminal end of bacterial alkaline phosphatase in order to produce a general "one-step" immunostaining reagent. (11). The 6His peptide domain is a widely used peptide fusion domain included in many expression vectors. Recombinant proteins "fused" to this domain can be readily purified by IMAC chromatography.

The scFv domain of the anti-6His scFv-phosphatase fusion product was cloned from a monoclonal hybridoma cell line that produced a mouse monoclonal antibody to 6His (11). The alkaline phosphatase gene was cloned from *E.coli* JM83 and fused to the 3' end of the scFv VH gene fragment. This scFv-phosphatase fusion product from bacterial crude lysates was found to give very strong and specific signals when assay against the antigen (citrate synthetase-6His) in standard ELISA assays as well as in the BIAcore assay (biomolecular interaction assays - Pharmacia AB Biosensor).

ScAb-M13 g3p Fusion Product

ScFv or ScAb are typically cloned as fusion products to M13 g3p. ScFv-g3p fusion products or Fab-g3p fusion products (12) are a fundamental elements of the antibody-M13 phage display technology (Pharmacia Biotech. ScAb-M13g3p or ScAb-M13 phage (scFv antibodies displayed on phage particles) are readily been used in the detection of botulinum toxins and WEEV antigens by ELISA or western blot (13, 14). An anti-6His scFv (3D5) antibody cloned as a fusion product to the N-terminal end of M13 phage g3p has also been successfully used in BIA analysis as

well as in two-step immunostaining procedures for ELISA [i.e. used as the first (1st) antibody reagent with anti-M13 HRP conjugate as the second (2nd) antibody reagent](11).

Fab-protein A Fusion Product

Recombinant FAb have been expressed as a fusion product with *Staphylococcal aureus* protein A (42Kdal) to form immunodetection reagent. The protein A domain is fused to the C-terminus of the Hc and the FAb heterodimer is formed when this fusion product is co-expressed with the Lc gene. When used in ELISA using biotinylated anti-human k chain antibody and streptavidin-HRP, the FAb-spA fusion product gave increased sensitivity in detection of the HSV-2 antigen compared to the regular recombinant FAb product.(15)

Open Sandwich ELISA Using ScAb

One of the functional disadvantages arising from the structure of many scFv antibodies is the often weak intermolecular interaction between the VH subunit and the VL subunit (16). This weak pair often creates unstable antigen combining sites. This can be remedied by using disulfide bonds between the VH and VL subunits to more rigidly stabilize the scFv structure. However this instability has been reported to be useful in the development of a novel "Open ELISA" technique (16).

Often either the VL domain or the VH domain alone has some antigen combining affinity. In this assay, the two scFv domains are kept separate (16). The VL domain is immobilized for the BIAcore analysis, and it is incubated with the antigen (hen egg lysozyme). Low measurable levels of antigen combining to with the immobilized VL were detected. Upon addition of VH-phage [VH displayed on M13 phage particle], pairing of the VL with the VH subunits occur and increased antigen-binding is measured (Figure 1). The increased binding to the antigen is the result of the

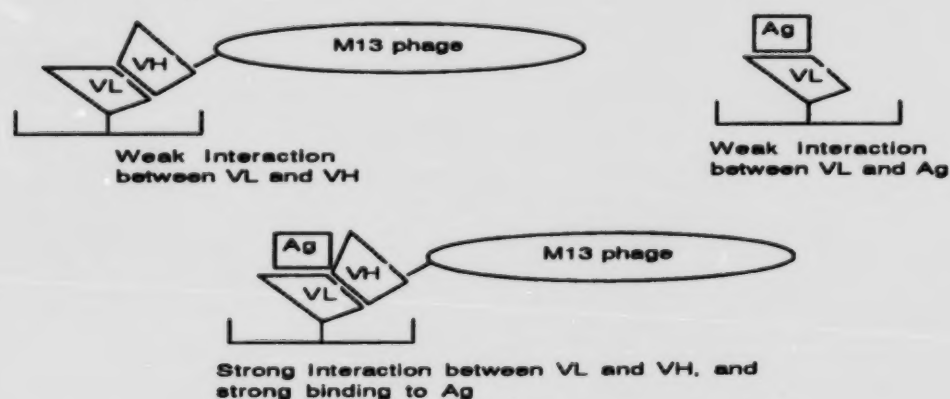


Figure 1. Stability of VL, VH and Antigen complexes in the "Open Sandwich ELISA" assay (16).

complete formation of an antigen combining site. The pairing of the VL with the VH subunit was correspondingly stabilized by the presence of the strongly bound antigen.

Work on application of scFv and other recombinant antibodies reported in the literature have been primarily focussed on human medical therapy. This is because current sources of animal antibody are inadequate and technical limitation for medical therapy have been reached. Current work has been driven by the potential of recombinant antibodies to perform at levels beyond the current technological limitations (i.e. provide high-affinity recombinant human antibodies).

In contrast, the current status of immunodetection technology has generally been deemed adequate using the current sources of animal antibodies. Indeed, the antibody source and immunodetection technology have co-evolved, so that immunodetection methods are optimized for antibody from animal sources. The greater complexity of recombinant antibody technology and higher cost in preparation of recombinant antibodies, have likely slowed work in this direction. However the unique properties of recombinant antibodies (i.e. improvement of binding by mutagenesis and genetic fusion to other useful protein partners) will likely facilitate future development of new immunodetection techniques so that recombinant antibodies are not just simply applied to older technologies. Work on recombinant antibodies for current immunodetection methods have primarily been focussed on those with broad applications (i.e. universal reagents employing the 6His tag or protein A tag) and those which can be readily used in conjunction with currently available immunodetection reagents.

As recombinant antibody technology improves, costs decrease and new immunodetection technologies evolve, the use of recombinant antibodies (scFv, Fab and antibody fusion products) in the field of immunodetection and immunodiagnosis will increase.

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Review of Immunobiosensor Technology

The great difficulty in identifying biological molecules come from their general complexity

1. 2. All proteins or nucleic acids are compounds of C, H, O, N and P (ie. amino acids or nucleotides) although they are structurally very different. Analysis by simple reduction to % elemental composition (i.e. C, H, O, N or P; or even % amino acid or base composition) is not useful. Techniques based upon spectroscopy, NMR (nuclear magnetic resonance) or chromatography are hampered by the large and complex nature of biological molecules often comprised of repetitive units (i.e. sugar units, amino acids, nucleotides). It is the higher ordered and complex structure of biological molecules which make their identification difficult 1,2.

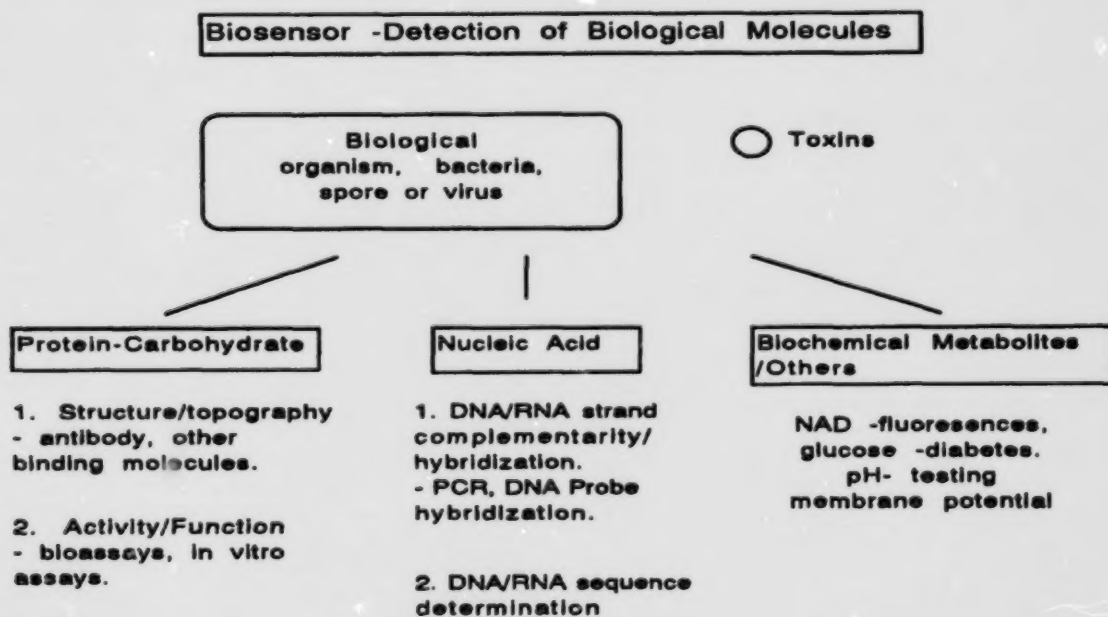


Figure 1. Detection of Biological Molecules and Organisms.

Proteins, carbohydrates, nucleic acids and fatty acids are biological molecules unique to biological organisms (Figure 1). The functional nature of biological molecules requires them to physically interact or bind to other biological molecules. Biomolecular interactions are a defining characteristic which typifies the behavior of most biological molecules. Current biodetection strategies take advantage of these naturally occurring interactions 3,4,5. Typically one binding molecule (i.e. an antibody or ligand, or DNA probe) is tagged so that it is measurable and its

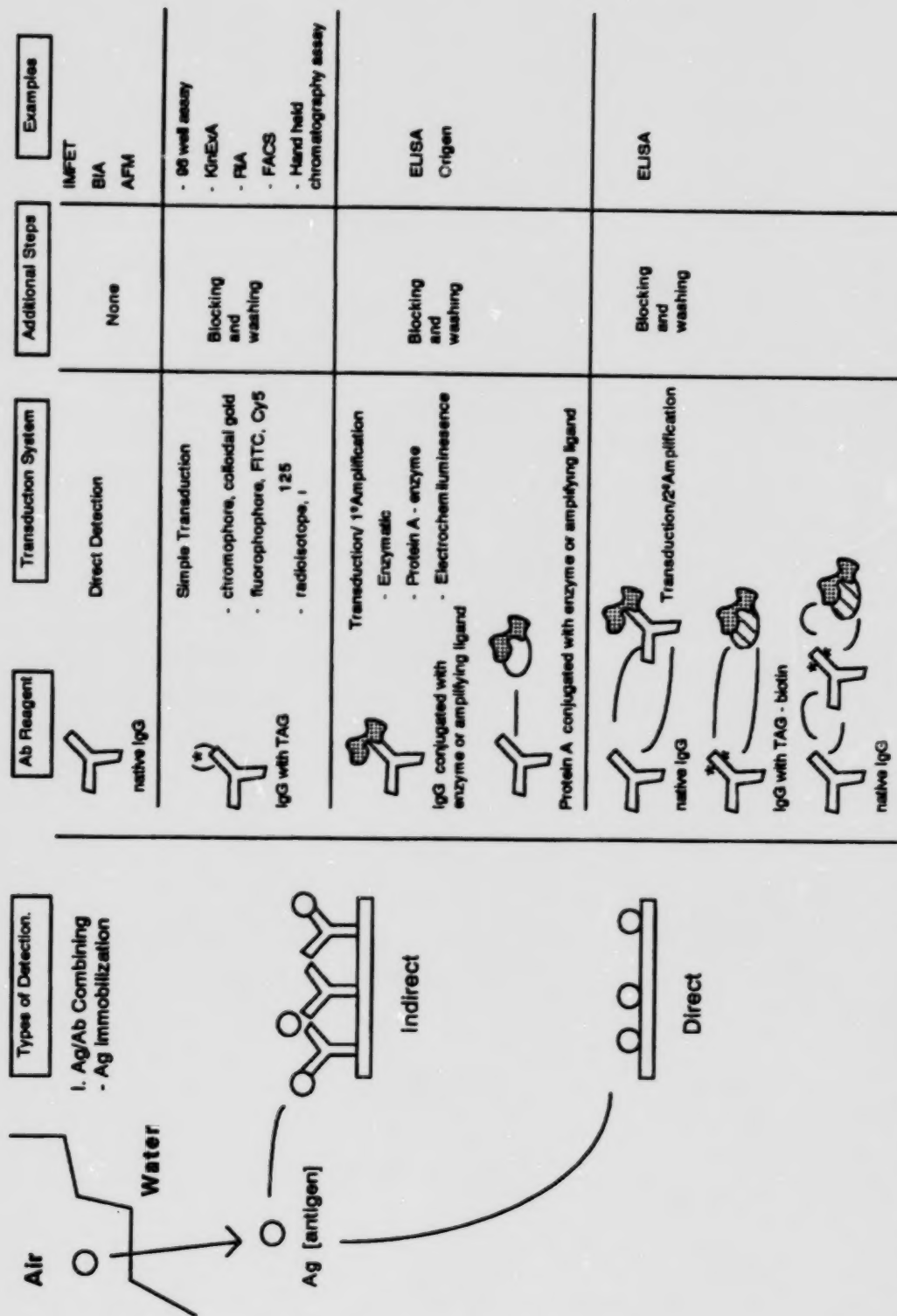
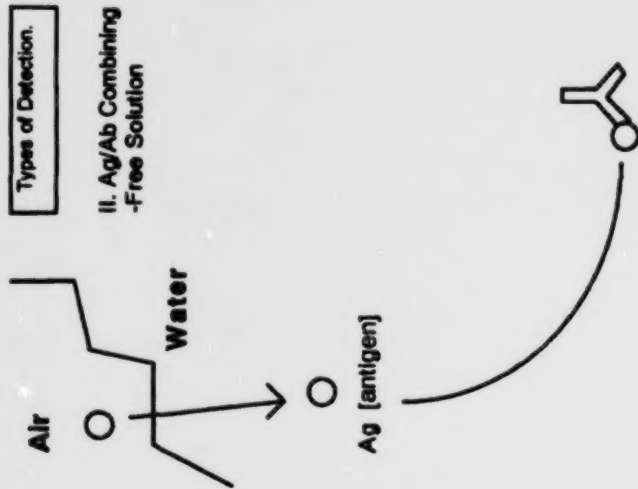


Figure 2. Solid-phase Immunosensors based upon Ag/Ab Combining using one immobilized partner.



Types of Detection	Ab Reagent	Transduction System	Additional Steps	Examples
II. Ag/Ab Combining -Free Solution	 native IgG	Direct Detection	None	UV-CE
	 IgG with TAG	Simple Transduction - fluorescent/Cy5	None	LIF-CE FACS flow cytometry
	 IgG conjugated with enzyme or amplifying ligand	Transduction/ 1 st Amplification	Not Developed	Not Developed
	 native IgG  IgG with TAG - biotin	Transduction/ 2 nd Amplification	Not Developed	Not Developed

Figure 3. Liquid-phase Immunosensors based upon Ag/Ab Combining and Detection in Free-Solution.

interaction with its binding target is then measured. The challenge of the biosensor field is to interface naturally occurring biological interactions with signal transducers (photometric, mechanical, or electrical) in order to determine whether or not a biological interaction is occurring ^{3,4,5}.

A biosensor is an analytical device which uses biologically active molecules to detect biological or chemical species directly without the need for complex sample preparation. The biologically active molecules may be enzymes, an organelle, a membrane component, a cell, an antibody or antigen, or whole slices of tissue. A method of detection employing a transducing system is usually used to convert a biochemical response into an electrical, photonic or mechanical signal which can later be processed ^{3,4,5}. If no aspect of the native interaction can be measured, then a transducer tag (which can be measured) is used to label one of the components of the biological interaction.

Immunosensors are a specific antibody-based biosensors which utilize antibodies in the identification of biomolecules and organisms. The general format for antibody-based detection systems, involves the capture of aerosol antigen into an aqueous environment where the antibody and antigen combining interaction can occur. There are two design formats used in the manufacture of immunosensors. The first are "solid-phase" immunosensors which requires the immobilization of the antigen or the antibody/antigen complex onto membranes, synthetic beads, plastic surfaces or any other material surfaces prior to measurements of the transduction signals (Figure 2). The second are "liquid-phase" immunosensors which allows antibody/antigen immunocomplexes to form in free-solutions and be measured in free-solution (Figure 3).

SP(Solid Phase) -Immunosensors

The fundamental interaction of antibody-antigen combining is the same in all immunosensors. The major differences are on how this fundamental interaction is "transduced" into a measurable signal and how that signal is amplified. The simplest solid-phase immunosensor measures the antibody/antigen interaction with only the native antibody (unlabelled or conjugate). SPR (Surface Plasmon Resonance) or BIA (Biospecific Interaction Analysis) ⁶⁻¹⁰, IMFET ⁵ Immuno Field effect Transistors) and AFM (atomic force microscopy)¹³ immunosensors are three examples.

BIA Immunosensors ⁶⁻¹⁰

The introduction of a biosensor based on surface plasmon resonance (SPR) in 1990 represents an important landmark, since it is the first time a major innovation had focussed on the elucidation of biological activity rather than structure. Biosensors based on SPR have been labelled "Biospecific Interaction Analysis (BIA)" immunosensors, and are revolutionizing the

immunoassay technology industry. In this technology antibody is immobilized onto the polished metal surface. The surface plasmon wave is an EM wave which propagates along the surface of a metal⁵.

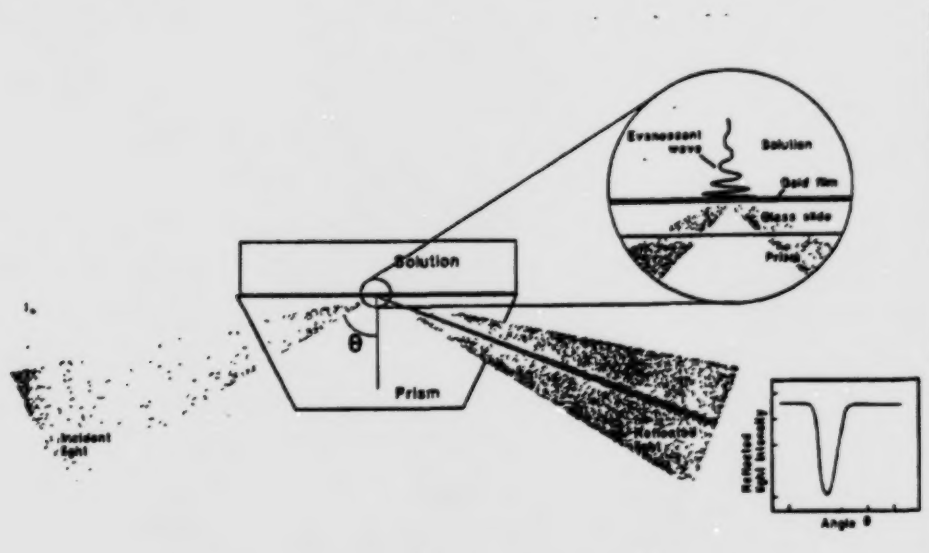


Figure 4. Surface plasmonon resonance (SPR) arises when light is totally internally reflected from a metal-coated interface between two media of different refractive index (a glass prism and solution in BIA), SPR is observed as a reduction in the intensity of the reflected light at a specific angle of reflection θ .

The resonance angle is very sensitive to variations of the refractive index of the medium bonding the metallized surface. Binding of the antigen to the antibody immobilized onto the surface can therefore be detected a a change in the resonance angle. This technique is very sensitive (<2ug/ml protein can readily be detected). Since all proteins have the same refractive index and since there is a linear correlation between resonace angle shift and protein concentration near the surface, changes in protein concentration at the surface due to protein-protein or protein-peptide binding can be measured accurately. The measurements using BIA analysis are done by monitoring protein-protein or ligand-receptor interactions by using changes in SPR measured in real time. This allows direct measurements of both the on and off rates of complex formation.

BIA technology makes it possible to find out which molecules interact, how many of them interact, how quickly and how stongly they interact, what their topographic distribution looks like, what the binding stoichiometry is, and whether or not conformational or allosteric changes occur upon binding³. BIA Immunoassay allows for faster characterization of monoclonal Ab's,

therefore the time for initial screening is drastically reduced and efforts can be concentrated on a few cell lines ³.

These are some of the advantages of SPR /BIA immunosensors:

1. Very little material required (1-10 μ g)
2. Low solution volumes (0.01 - 1mL) required.
3. Method is very fast (~10min)
4. No antibody-protein modifications are required (eg: labelling)
5. Interactions can be observed in complex mixtures
6. On rates and off rates are readily obtained
7. The system is useful over a wide range of protein concentrations
8. The system is quite sensitive
9. Allows measurement of affinity and kinetics of an interaction.

The main limitation of this technology is that sensitivity depends upon the molecular weight (MW) of the adsorbed layer which controls its optical thickness (low concentrations of small molecules (MW <250) such as haptens are unlikely to be measurable.

Other disadvantages of SPR are:

1. Antibody or ligand must be immobilized and this can potentially impede or enhance interactions.
2. Sensor chip must be regenerated under conditions which do not denature the immobilized ligand-protein

BIA immunosensor are available from various suppliers which have very different designs.

Biacore BIA Instrument ⁸

As the first company (Pharmacia Biosensor) to market a BIA instrument, Biacore actually changed it's name from "Biosensor", to ensure the association of their BIA instrument (called BIAcore) with their company. In this design, antibody or ligand is immobilized onto a gold coated sensor chips and test samples containing antigen or ligands flow over the reactive surface. This device measures of the binding of material to a sensor chip surface and monitors the formation of surface-bound macromolecular complexes in real time³. Specific ligands are immobilized to the sensor chip at low concentration through biotin-avidin interaction or covalently through amine, thiol or aldehyde chemistry ³. Using this device, association/dissociation kinetics of Ab combining to Ag (hen egg white lysozyme) can be readily determined¹⁰.

IAsys BIA Instrument ^{11,12}

The design of the SPR-based BIA immunosensor differs from the Pharmacia Biosensor product by using a cuvette style sensor sample chamber. Use of a cuvette requires the sample to be applied in and out the cuvette container. Antibody and ligands are coupled to the bottom of the

cuvette ^{11,12}. This device also incorporates an efficient vibro-stirrer (rather than a free flow system) to attain complete solution homogeneity within one second following sample addition thereby permitting accurate measurement of the earliest and most reliable rate data. Data is presented in the form of a real-time binding curve ¹¹. By contrast, flow-based biosensor systems present a parabolic leading front of sample for analysis. This effectively produces a concentration gradient of ligand in buffer which necessitates discarding at least the first 10 to 15 seconds of binding data.

The IAsys instrument differs from its Pharmacia Biosensor counterpart in two major respects: (i) the biosensor surface to which the antibody is attached forms the base of a stirred cuvette; and (ii) the refractive index change associated with antibody/antigen complex formation is monitored by resonant mirror technology ¹¹. A potential advantage of the IAsys cuvette system is its ability to provide equilibrium binding data simply by allowing the ligand solution to remain in the cuvette until a time-independent response is obtained ¹¹.

Applications of SPR -BIA immunosensors

BIA immunosensors can be used for the thermodynamic characterization of protein interactions because of its ability to accommodate stepwise additions of ligands (antibody and antigens) ¹². The pseudo-first-order kinetic analysis used for BIAcore sensorgrams are comparable to IAsys sensor traces provided that ligand uptake by the sensor surface is essentially without effect on the ligand concentration in the liquid phase. Second-order kinetic analysis has been developed to allow the treatment of data to be extended to the low concentration region in the event that the pseudo-first-order assumption ceases to be a valid approximation. Methods have been developed to determine values of the pseudo-first-order rate constants obtained in the absence and presence of a competing ligand. These values may be used to characterize the interaction between ligand and competitor in solution.

IMFET Immunosensors ⁵

This potentiometric-based immunosensor design is considerably less developed than SPR/BIA -based immunosensors. IMFET (immuno-field effect transistor) immunosensors operate on the principle of an accumulation of charge density at an electrode surface and formation of a significant charge potential. IMFET transistors could be produced by immobilizing antibody onto a membrane. This membrane transistor would remain polarized i.e. impermeable to the passage of charge and registers a particular electrical potential. Upon binding to antigen the membrane resistance is changed and measurements of depolarization can be used to measure antibody-antigen interaction.

AFM (Atomic Force Microscopy) Immunosensors ¹³

This microscopy-based technique measures the "height or layering" of the antibody antigen complex on a solid mica surface. Antibody would be first be immobilized onto a mica flake and AFM height information for antibody alone was measured. Upon combining to antigen, the AFM height of the immunocomplex increased height, indicative of antibody-antigen combination ¹³.

All subsequent solid-phase immunosensors measures the antibody/antigen interaction with the use of antibody labelled or conjugated with chemical groups or enzymes (Figure 2). The complexity of immunosensor schemes using labelled or conjugated antibody are greatly increased. These strategies require the matrix to "hold" the antigen/antibody complex in place while multiple steps involving "blocking and washing" are performed.

Simple Transduction SP-Immunosensors

Immunosensor using IgG labelled with a chemical transducer tags [i.e. a chromophore (eg. colloidal gold), fluorophore (fluorescein or Cy5) or a radionuclide (¹²⁵I)] generally involve immobilization of the antigen directly or indirectly onto the matrix surface (plastic plates, bead polymers or membrane polymers) ⁵. Nonspecific binding sites on the matrix surface are blocked using nonspecific protein blockers (BSA or casein) and the "transducer" antibody (i.e. the antibody with the chemical tag) is bound to the immobilized antigen. After several washing steps, immobilized transducer molecules can be measured directly [i.e. fluorimeters (fluorescein), visual inspection/densitometers (colloidal gold) or radiometers (¹²⁵I)]. They can also be release from the matrix with denaturants and measured using other analytical separation technologies (i.e. electrophoresis, HPLC chromatography etc.). Immunosensors based on this technology have been designed around various formats.

96-well Plate Immunoassay

One of the most common formats for this type of assay involve the use of the 96-well plastic immunoassay plates currently available on the market ^{1, 17, 18}. Antigen is immobilized at the bottom of the plastic wells and subsequent reagent and washing steps are performed sequentially. Although these multi-step assays can be performed manually, a multitude of semi-automated (ELISA plate readers and washers, Molecular Dynamics, Biorad) and fully automated robotic systems are now currently available (Beckman, Biorad). For RIA (radioimmunoassay) using radiolabelled antibodies, 96-well plate formats with "breakable" wells are available; individual assay wells can be placed into vials and analysed in scintillation counters or Beta counters.

KinExA (Kinetic Exclusion Assay) Instrument ¹⁸:

The KinExA instrument (Sapidyne Inc.) is an automated format which utilizes a capillary flow cell

with a filter-mesh located in the center of it to trap immunoparticles (polymethyl methacrylate beads). This capillary flow cell is embedded in an optical lens with an epi-illumination filter fluorometer mounted to detect fluorescence at the location of the trapped immunoparticles. In a typical assay, antibody and antigen are premixed in a mixing chamber then contents flow past the immunobeads. Antigen/antibody complexes and free antigen flow quickly past the plug of immunobeads (coated with antigen). Exposure of the Ab-soluble ligand complex to the immobilized ligand was sufficiently brief (240ms) to ensure negligible dissociation of the soluble complex during the time of exposure to the beads ^{18, 19}. Free-unreacted antibody binds to the immunoparticles. Following a wash step, a fluorescently labelled-second antibody which is specific to the first antibody, is used to stain the free-unreacted antibody bound to the immunoparticles. Following a wash step, fluorescence is then measured to determine rate constants and affinity of unlabelled biomolecules. This instrument is also amenable to other "sandwich or competition" assay formats.

This computer-controlled system is automated and uses negative pressure to operate and an in-line degasser to reduce bubbles that may cause erratic results. The manufacturers claim that results can be obtained in 2-15 minutes and give results comparable to ELISA. The use of antigen immobilized on the immunoparticles creates an effective high concentration of antigen (on solid phase), and uses low concentrations of unreacted antibody, therefore the reaction is fast and linear. This instrument reports to be able to detect free unbound antibody to the 10 picomolar range and can be used to determine antibody equilibria constants ^{18,19}.

Hand Held Chromatographic Assay

This is a common format for pregnancy testing kits which are commercially available at all pharmacies. In this format, reagent antibodies are immobilized as a fine line or a dot onto a chromatographic substrate. Antigen in solution is added to the sample chamber mounted at one end of the device. Antigen combines with the "transducer" antibody (labelled with colloidal gold, nickel or other chromophores) in a sample chamber and the immunocomplexes migrate by capillary force through the chromatographic substrate. The chromophore-labelled immunocomplexes are trapped by second antigen-specific antibody immobilized in the form of a line or dot. Color is concentrated onto these areas and positive detection manifests itself as a colored line or dot. Often control antibodies specific to the chromophore-labelled antibody are included as positive controls for this chromatographic format. The overall length of time to perform these assay is 10 to 15 minutes.

FACS (Fluorescence activated cell sorter) SP-Immunosensors

Flowcytometers are devices which separate and count single-cells or small particles in

solutions by generating a flow of these particles through a narrowing along the length of a thin flowing capillary ¹⁴. Detectors mounted in this region; 1. count the particles, and 2. measure light scatter and fluorescence of each individual particle as they pass by.

In this immunoassay, antigen is first immobilized onto polymer beads and reacted with fluorescent antibodies. Suspensions of the these beads are separated and counted by FACS, and a positive signal is the identification of a subpopulation of fluorescently labelled particles.

Primary (1st) Amplification/Transduction SP-Immunosensors

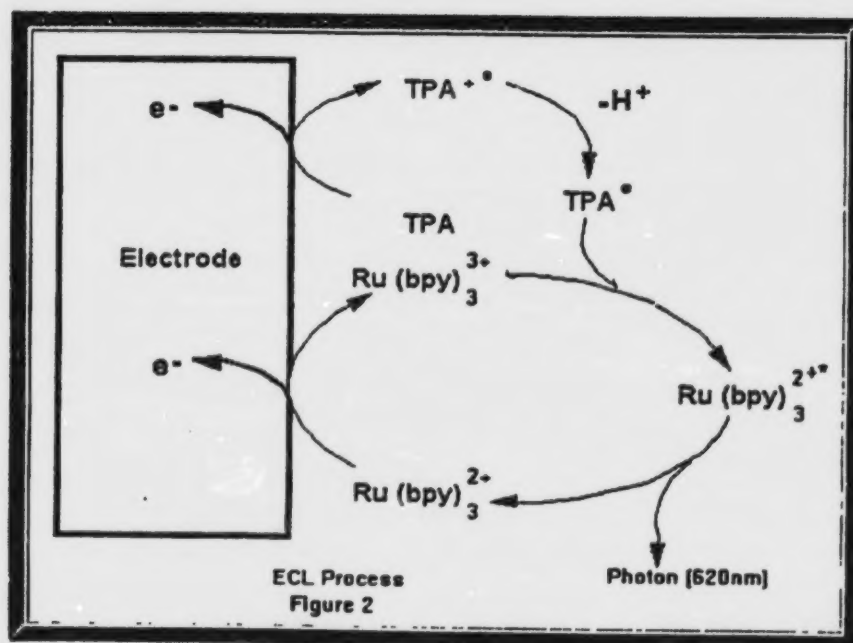
Simple transduction immunosensors use antibodies labelled with a single unit of "transducer signal"/ antibody molecule (ie. fluorescence, color or radioactivity). To increase detection sensitivity, strategies have developed with amplify the signal. Such techniques involving coupling of enzyme activities (e.g. alkaline phosphatase, horseradish peroxidase, urease e.t.c.) or chemical ligands which allow signal amplification (e.g. electrochemiluminescence), to the antibody.

ELISA¹⁵

ELISA (Enzyme-linked immunosorbant assay) is probably the most widespread, economical and reliable methods for immunodetection currently available ^{1, 15, 16, 17}. The classic technology involves the immobilization of antigen onto 96-well ELISA plates, followed by blocking and washing steps. The antibody-enzyme conjugate is reacted with the immobilized antigen and colormetric enzyme substrates added (e.g. ABTS, TMP, BCIP/NZT). Since the enzymes have a catalytic activity which converts substrate into a colored substance, the signal produced from an single antibody-conjugate molecule is amplified. Sensitivity using this technique has been greatly enhanced by the use of chemiluminescent substrates (e.g. ECL, supersignal™, CSPD) and devices which measure the light generated. As previously mentioned, these assays can be performed either manually or automatically using the multitude of semi-automated devices and fully automated robotic systems which are available. The basic technology utilizing 96-well ELISA plates as the solid phase can also be readily transferred to other solid phase supports i.e. polymer beads (immunoprecipitation assays) or synthetic membranes (e.g.immunodot blots or western blots using nitrocellulose, PVDF).

Origen Detection System (Igen Inc.)^{20, 21}

This immunoassay system is based upon electrochemiluminescence.^{20, 21} In this format, antibody is conjugated to a ruthenium ion (RU) bound in a chelate of tris-(bipyridine). Low voltage triggers a cyclical oxidation and reduction reaction of the ruthenium metal ion which generates light. A second reaction component , tripropylamine (TPA) present in excess is consumed in the oxidation process and the ruthenium chelate conjugated to the antibody, is recycled (Figure 5).



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Figure 5. Electrochemiluminescence Process.

This cycling process intensifies and amplifies the electrochemiluminescent signal. In a the "sandwich" format of this assay, antigen is combined with antibody labelled with ruthenium chelate (typically 1 to 25 molecules/antibody molecule). Paramagnetic beads coated with a second antigen-specific antibody are added, and immunoprecipitate/paramagnetic beads are pumped from the reaction vial through the detection cell and onto a platinum electrode. At this location, light production which occurs from the oxidation reactions over a period of 1 second is detected by a photodetector. With this highly sensitive format, detection of sub-picogram levels protein are possible using even low affinity antibodies.

Secondary (2nd) Amplification Transduction Immunosensors

Strategies for further amplification of the transduction signals have been devised utilizing a

primary antibody (native or chemically-modified e.g. biotinylated) and a secondary immunostaining conjugate (i.e. anti-primary IgG antibody or streptavidin protein conjugated to either an enzyme or amplifying ligand). In this strategy, multiple copies of amplifying conjugate are added to the each primary antibody in order to increase the transduction signal. Potential assay formats for this strategy are the same as for primary (1st) amplification transduction immunosensors. Major differences are the increased number of steps involving incubation and washing of assay plates. It is possible to increase the number of immunostaining reagents in the sequence to further increasing the signal output [eg. Antigen binds (1) primary antibody, which binds (2) biotinylated second antibody, which binds (3) streptavidin-enzyme conjugate which is then reacted with the substrate]. However caution should be taken since increasing the number of immunostaining reagents risks often higher non-specific background in the assay.

LP (Liquid Phase) Immunosensors

Solid-phase (SP) immunosensor typically utilize immunocomplex immobilization onto a solid surface in order to allow contaminants or excess antibody to be washed away ²². Liquid phase (LP) immunosensors must separate immunocomplexes from contaminants and excess antibody without an immobilization step. Antigen/antibody complexes are formed in free solution and transduction signals are measured from antigen/antibody complexes in free solution. In the basic liquid phase immunosensor, antibody is combined with antigen to form an immunocomplex in solution. The immunocomplexes in the solution are separated from contaminants, excess antigen and excess antibody by some a chromatographic technique (e.g. capillary electrophoresis, flow cytometry, or HPLC) or ultrafiltration medium. The immunocomplex is then identified by the chromatographic characteristics using some transduction system. Capillary electrophoresis is the most prominent instrument used in LP immunosensors.

Simple LP-immunosensors

Current designs of LP-immunosensors using unlabelled antibody have very limited application, since no measuring parameter or device is available to 1) detect biomolecular interaction between antigen and antibody, or 2) distinguish between antigen/antibody immunocomplexes and contaminating proteins in free solution ^{22, 23}.

UV-CE (Capillary Electrophoresis) ^{22,23}

CE (Capillary Electrophoresis) is electrophoresis performed in thin glass capillaries (10-200 μm id) under very high voltages (200-400 volts/cm length of capillary). The forces acting to separate the protein samples are a combination of electrophoretic migration (based on net charge) and electroosmotic flow, EOF (bulk flow of electrolyte buffer); so protein separate according to charge to mass ratio. Depending on the length of the capillaries, typical sample separation times can

range from 5 minutes to 20 minutes ^{22,23}.

In the CE-based immunoassay, antibody/antigen immunocomplexes formed in solution can be separated from unreacted antibody or antigen by differential migration rates using capillary electrophoresis. Protein peaks are measured spectrophotometrically (OD₂₈₀) as they passing a UV detector at a fixed location on the capillary length. Immunodetection of the immunocomplexes by UV-CE detection is only possible if pure antibodies and antigens are used. This technique is ineffective for analysis of impure antigen samples.

Simple Transduction LP-Immunosensors ²²⁻²⁶.

The only useful and effective LP-immunosensors available to date uses fluorescently-labelled antibody and CE technology. Using antibody conjugated with fluorophores (e.g. fluorescein or Cy5) allows antibody/antigen immunocomplexes to be distinguished from contaminating proteins and unreacted antibody in the antigen sample.

LIF-CE-immunosensor ²²⁻²⁶.

Using fluorescently labelled IgG [i.e. fluorescein or Cy5] , complex antigen samples can be assayed using a LIF-CE (laser-induced fluorescence capillary electrophoresis). Since this detection instrument is setup to detect only fluorophores, only peaks corresponding to the antibody and antibody/antigen immunocomplexes will be detectable. A positive transduction signal is marked by the appearance of a unique protein peak corresponding to the antibody/antigen complex. Using LIF technology increases the sensitivity of detection 1000X above that of standard UV detection. LIF-CE instruments are available through several commercial companies (Beckman, Hewlett Packard and BioRad). Additional accessories to the basic CE apparatus include LIF attachments (488 argon ion laser or 635 semiconductor laser), and mass spectrometry adapters.

LIF-CE microchip immunosensor

Efforts by DRES are underway to miniturized the LIF-CE immunoassay onto a microchip. These CE-microchips containing extensive arrays of microchannels and electrode arrays which will allow electrokinetic pumping of reagent from one part of the chip to the other. The objectives are to perform the LIF-CE immunoassay using ovalbumin as a simulant BWA within 5 minutes.

FACS -based Immunosensor ¹⁴.

FACS (fluorescence-activated cell sorter) flow cytometry functions as a transduction LP-immunosensor when particulate antigen (e.g. virus, spores or bacteria) is being tested. Fluorescent antibody are incubated with antigen suspensions and then processes through a FACS flowcytometer.

Amplification/Transduction LP-Immunosensors

Because of the free-flowing and dynamic nature of LP immunosensors, use of currently

available enzyme or chemical signal amplification-based transduction systems is not possible. Colormetric or chemiluminescent substrates can diffuse throughout the system or away from the micro-locations of immunocomplexes and misrepresent the chromatographic characteristics of an antibody/antigen immunocomplex.

Summary

Immunosensors which can be grouped into two general categories, 1) SP (solid-phase 2) LP (liquid-phase) utilize a wide variety of transduction systems to detect antibody-antigen combining. Of the common immunosensor formats reviewed, literature and product specification all essentially reported detection limits of down to 10^{-12} M. By far the largest category are the SP-immunosensors.

All solid-phase immunoassays have problems in reproducible immobilization of reactants to material surfaces. In particular formats where antibody/antigen combining occurs with one immobilized partner (e.g. BIAcore) reaction kinetics are slower. Some SP-immunosensor formats overcome this problem by first reacting antibody and antigen in free solution prior to immobilization of the immunocomplex (e.g. KinAx). Another problem with SP-immunosensors is nonspecific protein binding to the solid surfaces. The elimination of contaminating proteins and unreacted reagent antibodies is a major issue with transduction SP-immunosensors which use antibodies conjugated with "transducer" molecules (e.g. KinAx and ELISA). Often blocking steps and washing steps are essential to obtain clear transduction signals for SP-immunosensors. This is not a problem for simple SP-immunosensors (e.g. BIAcore and IASys). Indeed an important advantage of BIA technology is that relatively impure antibody samples can be readily assayed.

LP-immunosensors as represented by LIF-CE technology or FACS flowcytometry are less affected by the presence of contaminating proteins and the need to remove unreacted antibodies conjugated with "transducer" molecules. Since there is no solid phase, antibody antigen combining kinetics in free solution is fast. In addition no time or effort is required for additional steps for immobilization, blocking or washing steps. In this regard, LIF CE is significant alternative. The problems associated with LIF-CE include a need for a strategy for performing multiple sample analysis, and optimization of CE conditions to resolve immunocomplexes from unreacted antibody. Another problem with this technique is that it requires highly purified antibody reagent (direct assay) or antigen (competitive assay) that must be homogeneously labelled with fluorophore.

Integration of Analytical Technologies ^{29, 30}

Immunosensors described here represent solitary technologies which are amenable to integration with other technologies to establish more robust detection systems. The nature of BIAcore or LIF-CE immunosensor formats facilitate easy integration with mass spectroscopy ²⁷.

28. Antigen samples identified by the immunosensor can be readily electrospray ionization for mass spectroscopy. Resulting antigen mass spectrographs can be compared to information in large databases to identify the antigen. Another powerful technology that can be integrate with immunosensors is PCR. In immunoPCR or immunocapture PCR, the antigen must be associated with nucleic acids (i.e. virus particles, spores or cells) ^{31, 32}. Most SP-immunosensors and FACS-based LP-immunosensors affinity purify antigen during their operations; trapped product can simply PCR-amplified and analyzed by electrophoresis. Data obtained can be compared to information in PCR-based detection database to positively identify the unknown antigen.

Many of the immunosensor technologies reviewed above may be potentially useful in helping DRES to further develop it's BWA detection system. Use of LP-immunosensors may be more amenable for integrating a variety of technologies (Figure 6) ³³.

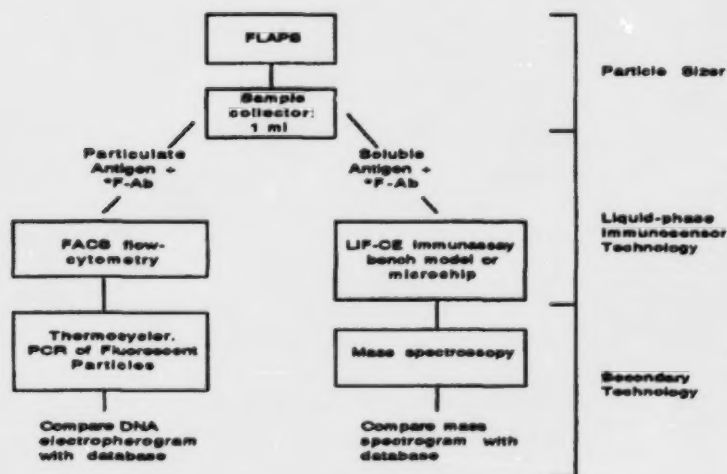


Figure 6. Proposed use of Liquid-phase Immunosensors in Detection of Aerosol Pathogens and Toxins. The FLAPS (fluorescence laser activated particle sizer) device determines nature of atmospheric particles and the air sampler collects an antigen sample. The test sample is resuspended in 1 ml of buffer and aliquots are mixed with F*-Ab (fluorescently-labelled antibody e.g. GFP-scFv or fluorescein labelled mAb). Using a FACS flowcytometer, fluorescence-labelled particles are identified and collected for quantitative PCR analysis using diagnostic primers. Using LIF-CE, fluorescence-labelled immunocomplexes are identified and collected for mass spectroscopy.

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Introduction

Antibody based immunodetection is an important technology in the identification of biological antigens (e.g. toxins) and biological agents (e.g. viruses, bacterial spores and bacteria). An important source of antibody is through animal antisera and monoclonal antibodies. Advances in recombinant antibody technology (1-3) provide an alternative source of antibody which is amenable to genetic humanization (4,5) and genetic manipulation to obtain advantageous properties (6-8).

Radioisotopes, fluorescent/chemiluminescent molecules, and enzymes chemically linked to antibodies have been used as reporter molecules to detect/quantify Ab-Ag interactions (100-1). These chemical modifications are susceptible to batch-wise variations, produce a reagent that is heterogeneously labelled, and often lead to inactivation of the antibody binding site. Genetically engineered chimeric antibodies whose binding domain is fused to an enzyme or other protein capable of generating an amplified signal offer an alternative to chemical modification and eliminates the above disadvantages without incurring any loss of sensitivity. Current work on proteins with intrinsic fluorescent properties provides a unique opportunity to genetically tag useful scFv.

Fluorescence in the bioluminescent jellyfish *Aequorea victoria* is generated by the sequential activation of two photoproteins, aequorin and green fluorescent protein (GFP) (10, 16). Upon oxidation of bound luciferin in the presence of Ca^{2+} , aequorin emits blue light (470 nm) which excites GFP to fluoresce green (10, 16). The genetic fusion GFP/GFP mutants with scFv's may potentially provide fluorescence-based immunodetectors with useful assay reagents.

Green Fluorescent Protein (27 kDa) from the jelly fish species *Aequorea victoria* has been extensively used in the microscopy studies of intracellular expression in eukaryotic cells. When this protein is illuminated with ultraviolet light (365 nm), the protein gives off a green fluorescent light (508 nm). Mutants of GFP have been constructed which emit red and blue fluorescent light and/or emit a stronger fluorescent signal. The genetic fusion GFP and/or GFP mutants with scFv antibodies may potentially provide fluorescence-based immunodetectors with useful assay reagents. Advantages of using a scFv-GFP fusion product in immunodetection include:

1. Available purified scFv-GFP fusion product without need for chemical conjugation. Chemical modification can inactivate the antigen combining site of the antibody (unpublished data).
2. ScFv-GFP fusion product is homogeneously labelled (i.e. 1 fluorescent protein tag/scFv-binding domain.). Chemical conjugation using protein modifying chemical groups (eg. fluorescein isothiocyanate, fluorescein-5-maleimide) generate proteins modified with a ranging

number of fluorescein-labelled residues [i.e. one scFv molecule may be modified with three (3) fluorescein molecules while another molecule may be modified with only one (1).] This is particularly problematic in immunodetection strategies which use capillary electrophoresis (CE). In CE, chemically-labelled fluorescent antibody reagent occurs in multiple peaks due to the differing mass of scFv caused by differing numbers of fluorescein molecules conjugated to the different protein molecules (unpublished data).

3. Purified scFv-GFP fusion product would make stable reagents.

Here we report the cloning of GFPuv (Clontech) into the scFv-T7 expresssion vector pRSD12-8 to generate scFv-GFPuv fusion vector, pRSD12guv. scFv-GFP fusion antibodies were expressed in *E.coli*. In addition, we have obtained anti-ovalbumin monoclonal hybridoma cell lines and here report the cloning of one of these clone into scFv phage-display vector pCANTAB5E.

Results

rGFP Immobilized on PS-Beads can be Detected by FACS.

Work was performed to determine if GFP fluorescences could be used in the detection of 2-8 micron particles (bacterial-viral simulants) using a FACS (Fluorescence-Activated Cell Sorting). Two approaches were taken to fluorescently label polystyrene beads with recombinant GFP (Clontech).

In the first approach, recombinant GFP was chemically coupled to 3.54 micron beads using EDC [1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Methiodide] at a ratio of 23 rGFP molecules/bed (see Appendix A for calculations). After the reaction beads collected by centrifugation could be observed to be labelled with rGFP by inspection under UV-irradiation (Figure 1). Visually approximately 100% of fluorescences was associated with the bead pellet with essentially no fluorescence observed in the supernatant. Supernatant (up to 1/20 of the total sample) was analysis by western blot analysis to determine if any uncoupled GFP was retained in the supernatant (Figure 2). Western blot can readily detect 0.5 ug of rGFP; thus 10 ug of uncoupled rGFP in the supernatant would be detectable by western blot. No detectable rGFP in the reacted supernatant suggests that it contains less than 10 ug of rGFP (10% of total rGFP used in coupling reaction). Conservatively, estimating that 70-90% efficiency in chemical coupling, a coupling ratio of 16-21 rGFP molecules/bead was like achieved.

Dilutions of 2.5% GFP-beads (rGFP-labelled beads) were analyzed using the Skatron Argus 1.72 FACS apparatus. After calibration with standard fluorescent beads [B1 (1 μ) beads, or Fluorobeads(FITC-10 μ) beads], GFP-beads were analyzed using several filter arrays (Table 1).

Table 1. GFP Filter Arrays for Skatron Argus 1.72 FACS apparatus

<u>Filter</u>	<u>Wavelength (nm)</u>		<u>Rating For GFP</u>
	<u>Excitation</u>	<u>Emission</u>	
B1	396-440	470	fair
B2	390-490	520	good
UV	365	420	poor

Using filter B2, FACS spectrograms were obtained that generally indicated that the PS beads were poorly labelled with rGFP (1-5% fluorescent beads). The intensity of the fluorescence of the GFP-beads was also weak (Figure 3).

The second approach more accurately simulates the application of recombinant GFP-

antibody in FACS-based detection. Dilutions of rabbit anti-GFP antisera were combined with dilutions of rGFP and constant amounts Goat anti-rabbit PS beads (0.7 to 0.9 μ) [Figure 4]. The mixtures were incubated at ambient temperature for 30 minutes. After washing of the bead immunocomplexes with PBS, GFP-PS bead immunocomplexes could be readily detectable by FACS analysis using filter B. (Figure 4. sample A2). Intensity of GFP-labelled beads were dependent on the ratio of the three components in the immunocomplexing assay.

Recombinant GFP can be Detected under Electrophoresis Conditions.

Another important immunodetection method which may utilize recombinant scFv-GFP antibody is LIF-CE (laser-induced fluorescence capillary electrophoresis)-based immunoassays. GFP protein is resistant to low concentrations of SDS and can be subjected to SDS PAG electrophoresis without deleterious effects upon the molecules fluorescence (Figure 2). These results suggest that GFP and scFv-GFP would also be amenable to separation by LIF-CE and LIF-CE immunoassay.

Construction of pRSD12guv

The cloning scheme used to create the bacterial expression vector pRSD12guv is outlined in figure 5. A restriction map of this scFv-GFPuv construct is shown figure 6-A. Primers for PCR amplification of GFPuv were designed, based on the sequence of pGFPuv cloning vector (Clontech, GenBank accession #: U62636), and are shown in Appendix B. pGFPuv (Clontech) was PCR amplified with GFPuv 5' Not and GFPuv 3' Hind primers giving a product with the expected size of 738 bp as depicted in figure 7. PCR-amplified GFPuv DNA (727 bp after Not I/Hind III digestion) was cloned into the Not I/Hind III site of pRSD12-8 (hereafter designated pRSD12guv) and transformed into *E. coli* TG1 cells. Fourteen transformants were randomly picked and screened by restriction endonuclease digestion. Figure 8 shows six of the fourteen clones produced the expected 2940 bp and 1510 bp bands after Eco RI/Hind III digestion. The predicted sequence indicates there is a coding capacity for a 545 amino acid protein with a molecular weight of 60.6 kDa.

Expression of GFPuv and RSD12guv

pRSD12guv is an *E. coli* expression vector that expresses foreign sequences as fusion proteins with the 26.6 kDa GFPuv protein. The fusion product contains a 6-His sequence for IMAC purification and an enterokinase recognition site for detecting products with α -express mAb (Invitrogen Corp.). After purification, the 6-His sequence can be removed by enterokinase



Figure 1. Fluorescence of recombinant wildtype GFP under UV illumination. Microtube A contains 10 μ l of 244 ng/ μ l wildtype GFP and microtube B contains 50 μ l of supernatant from a crosslinking assay of GFP (244 ng/ μ l) with 3.54 micron polystyrene beads (Spherotech Inc.) using EDC (1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide). Tubes were illuminated with UV irradiation (254 to 365 nm) and photographed with polaroid 667 film/ yellow filter (for commassie blue-stained gels).

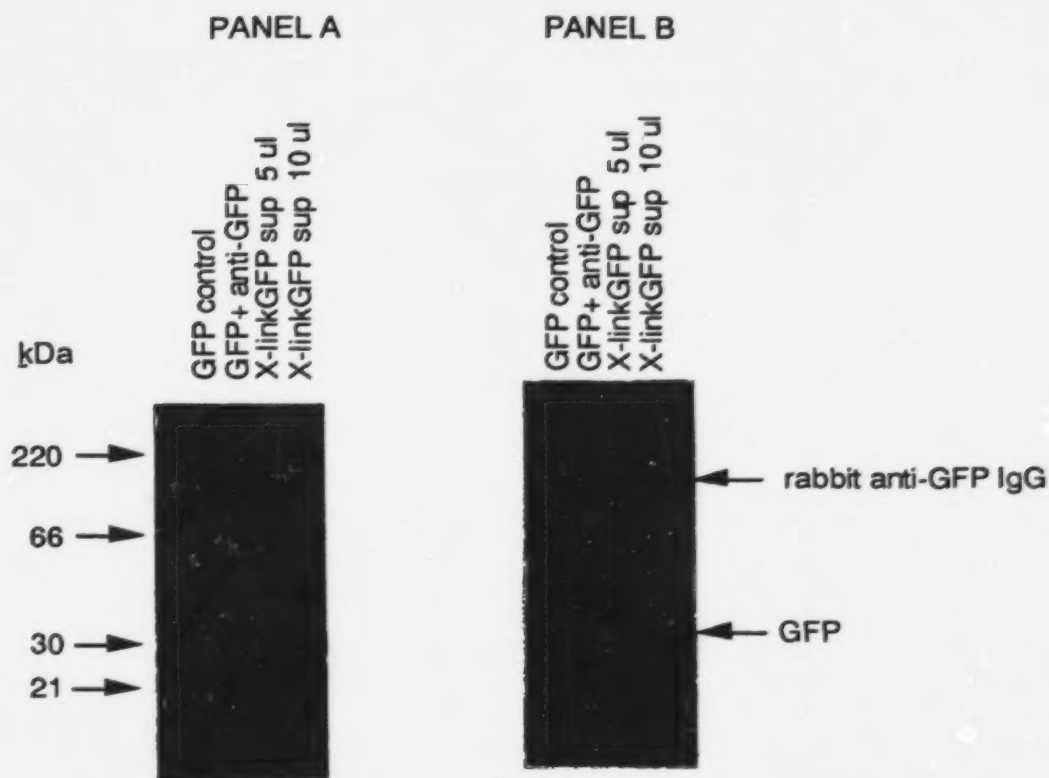
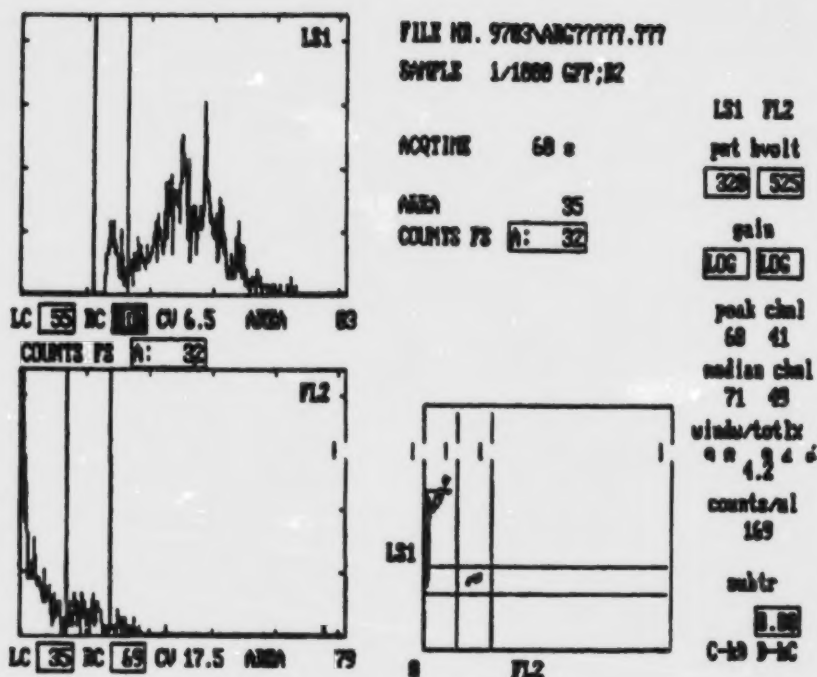


Figure 2. Analysis of recombinant wildtype GFP by SDS PAGE electrophoresis and Western blot. Samples of GFP mixed with loading mixture (1% SDS, 25% glycerol, 0.125M Tris pH 6.8, 0.1% bromophenol blue), were separated on SDS PAGE (15% polyacrylamide, 1% SDS). The gel lanes indicated are 1) *GFP control* - 5 ul of 244 ng/ul of GFP, 2) *GFP + anti-GFP* - 5 ul of 244 ng/ul of GFP + 5 ul of 1/500 dilution of rabbit anti-GFP antisera, 3) *X-link GFP sup 5 ul* - 5 ul of supernatant from a chemical crosslinking assay of GFP (244 ng/ul GFP reaction concentration) to 3.54 μ m polystyrene beads using EDC. 4) *X-link GFP sup 10 ul* - 10 ul of supernatant as described in 3). Panel A shows UV-induced fluorescence of GFP gel samples irradiated with UV (254 to 365 nm) as photographed with polaroid 667 film/ yellow filter (for commassie blue-stained gels). Panel B shows the western blot of the same gel immunostained with rabbit anti-GFP antisera (Clontech), goat anti-rabbit alkaline phosphatase and CSPD chemiluminescent substrate.

Panel A



Panel B

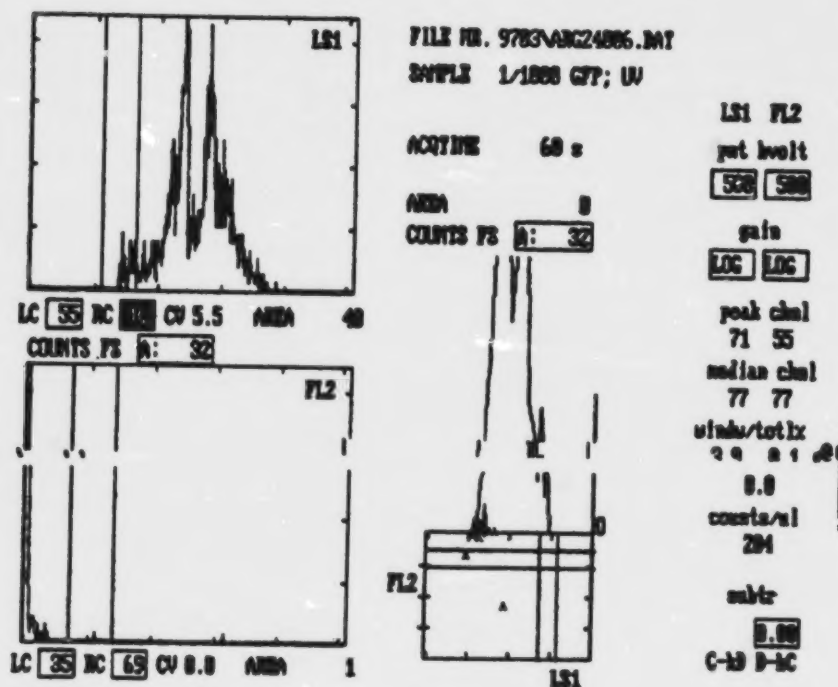


Figure 3. Detection of rGFP chemically coupled to PS-beads (pg 1 of 2).

Panel C

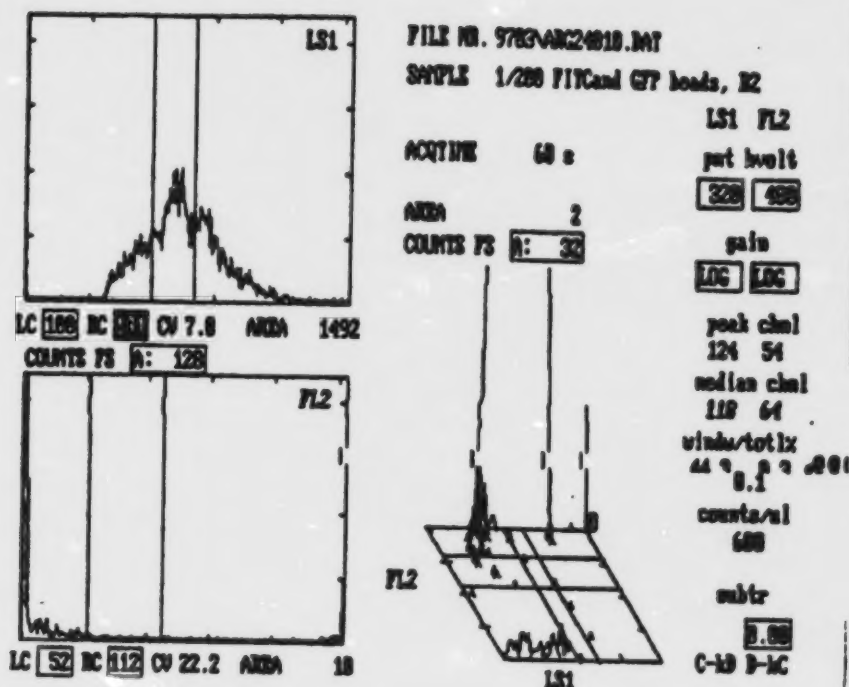


Figure 3. Detection of rGFP chemically coupled to PS-beads (pg 2 of 2). Recombinant GFP was chemically coupled to carboxyl PS (polystyrene) beads using EDC. Dilutions 1/1000 of 2.5% GFP-beads (rGFP-labelled beads) were analyzed using the Skatron Argus 1.72 FACS apparatus at a flow rate of 5 ul/ml using the B2 (Panel A) and UV (Panel B) filters. Panel C shows the analysis of mixture of 1/200 of 2.5% GFP-beads (3.54 μ) and 1/200 of 2.5% Fluorobeads(FITC-10 μ) beads using the B2 filter.,

Panel A.

	A: rGFP (1/20)	B: rGFP (1/200)
1. Rabbit anti-GFP (1/20)	A1. poor	B1. poor
2. Rabbit anti-GFP (1/200)	A2. good	B2. fair

Standard assay involves 50 ul of rGFP + 50 ul of Rabbit anti-GFP + 50 ul of Goat anti-rabbit IgG beads.

Panel B.

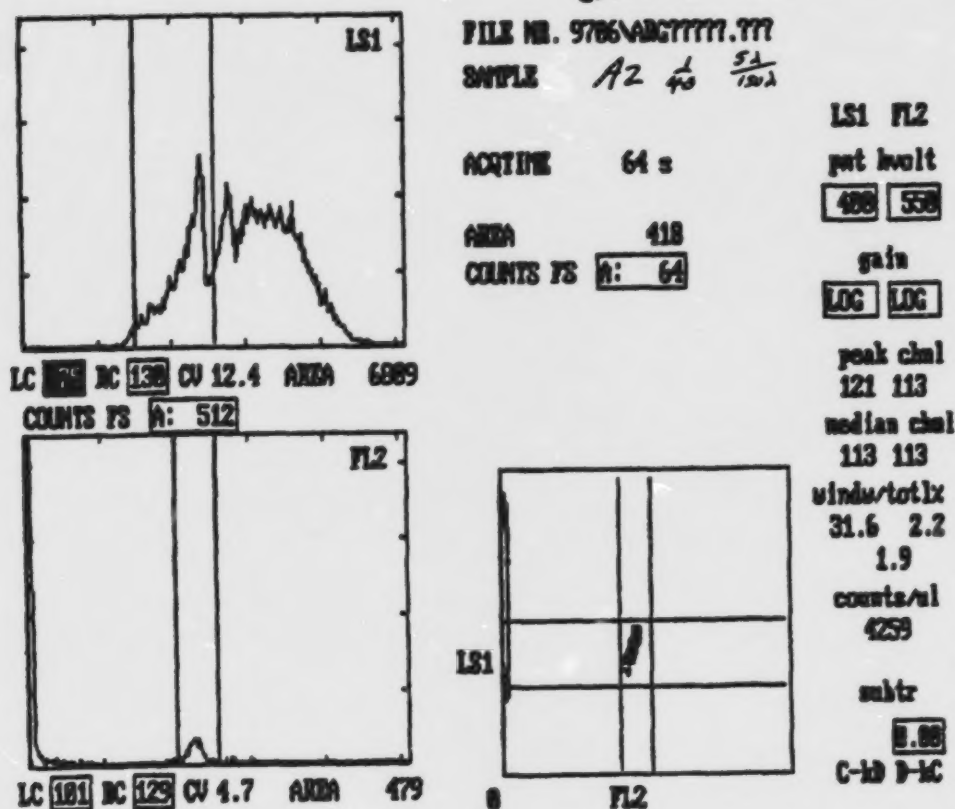
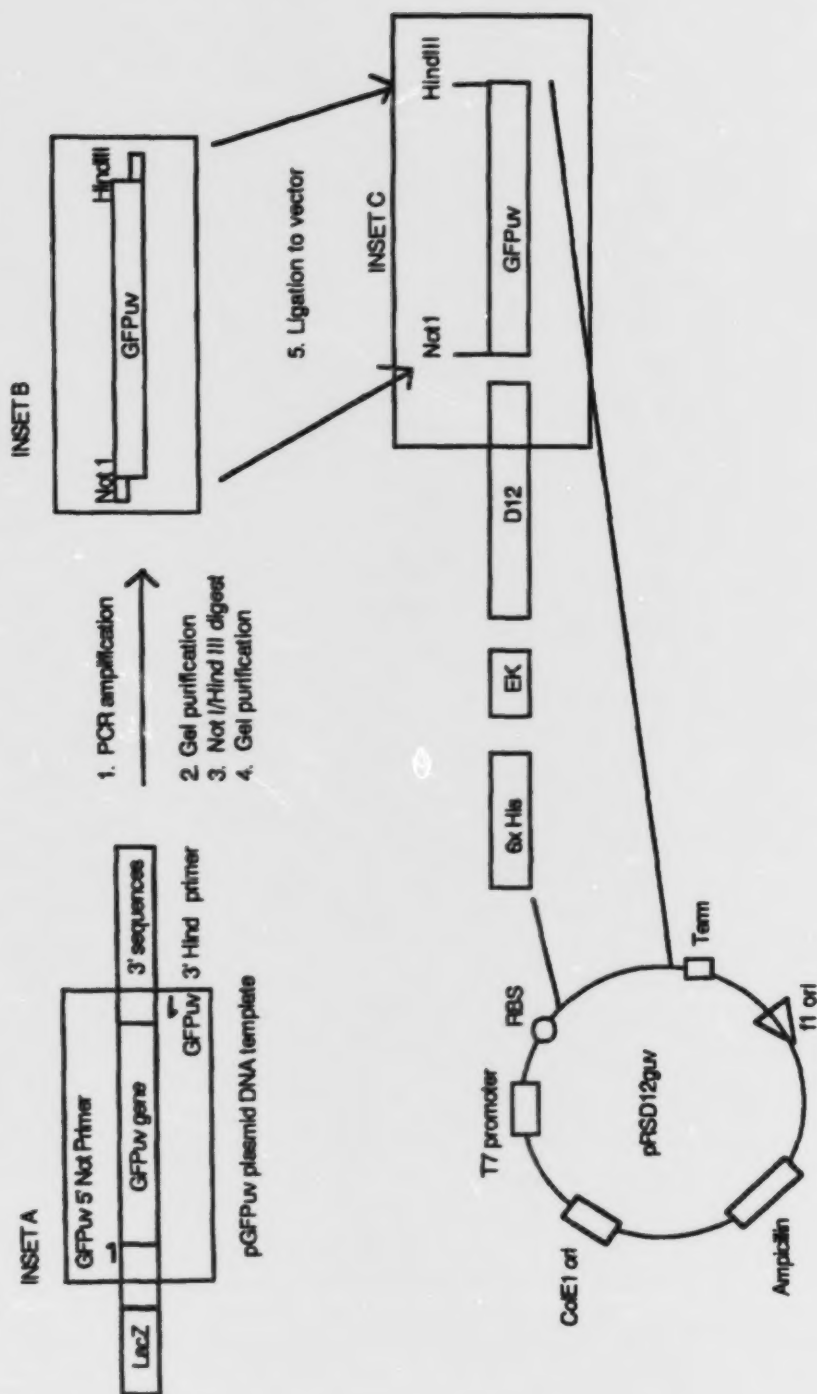
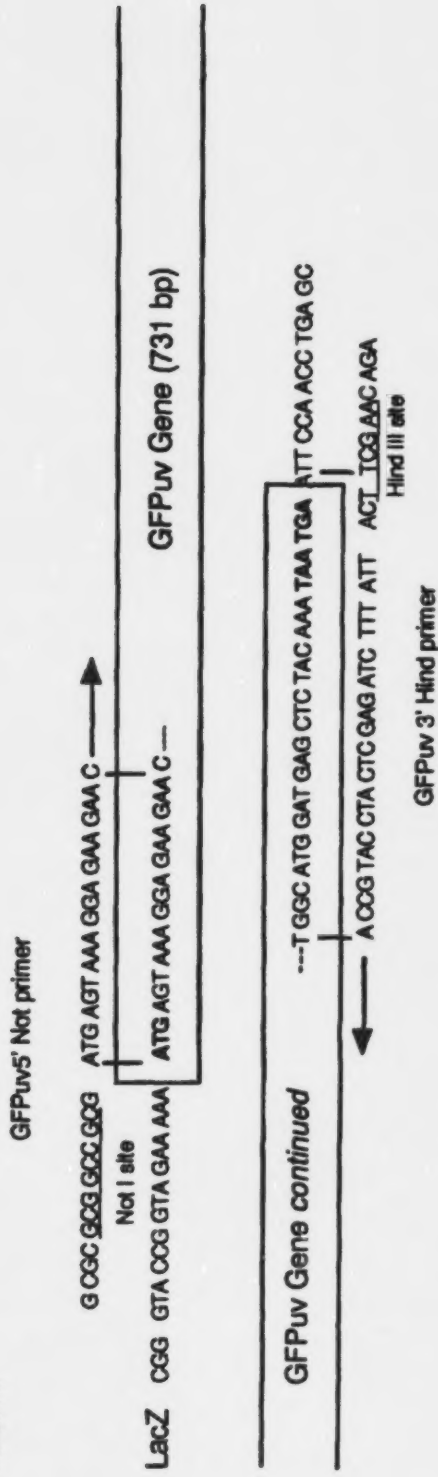


Figure 4. Detection of rGFP-IgG PS immunocomplexes by FACS.

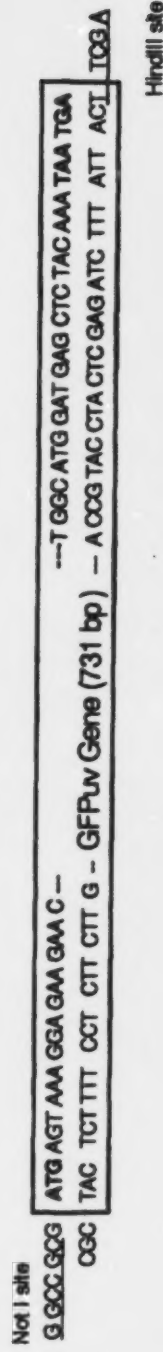
Panel A. Optimal Immunocomplexing of GFP-IgG PS beads for FACS Detection. Panel B shows a FACS spectrogram of experimental condition A2. Analysis was performed at a flow rate of 5 ul/min using the Skatron Argus 1.72 FACS apparatus and B2 filter.



Inset A:



Inset B:



Inset C:

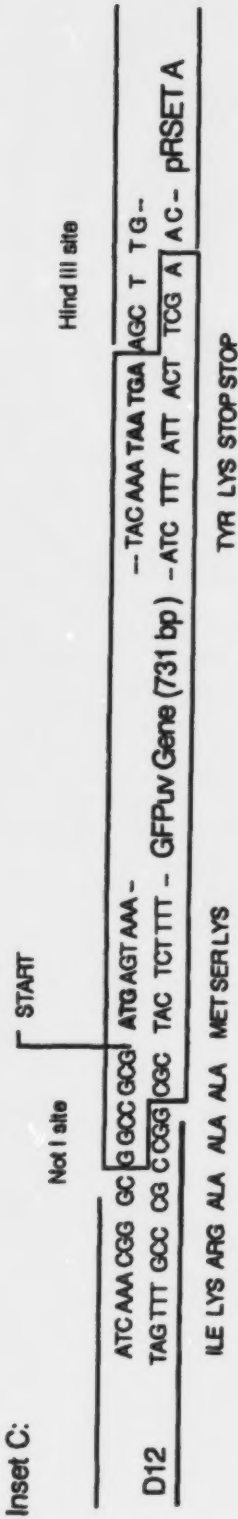
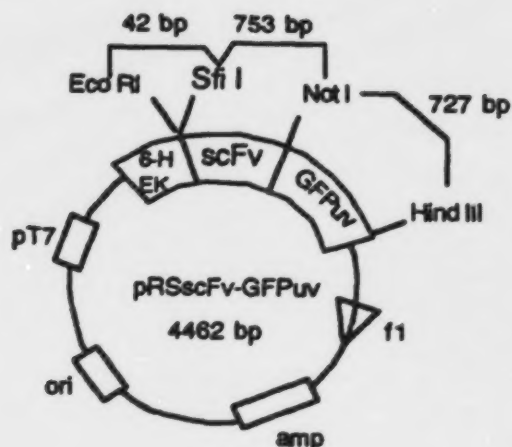


Figure 5 continued (2 of 2).

Panel A: Restriction map of scFv-GFPuv expression vector.



Panel B: Restriction map of scFv vector pCANTAB 5E.

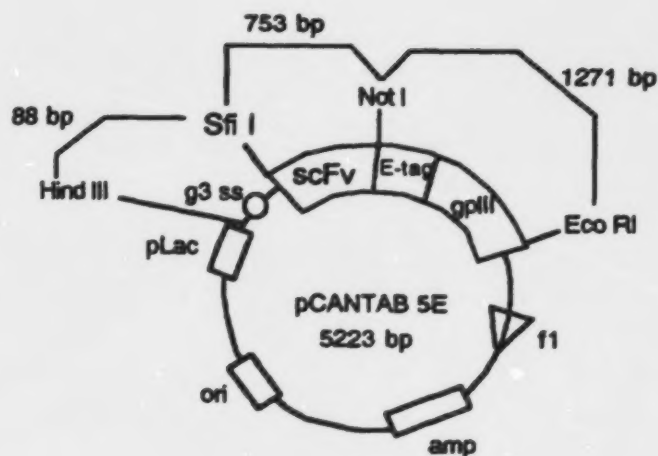


Figure 6. Restriction Maps of Generalized scFv cloning and expression vectors. Panel A depicts the restriction map of generalized scFv-GFPuv expression vector. Panel B depicts the restriction map of pCANTAB 5E (Pharmacia), a scFv cloning vector. pT7=T7 promoter, pLac=lac promoter, g3 ss= gpIII signal sequence, ori= ColE1 origin of replication, f1= f1 origin of replication, E-tag=peptide E, 6H-EK=6His affinity tag and enterokinase cleavage site.

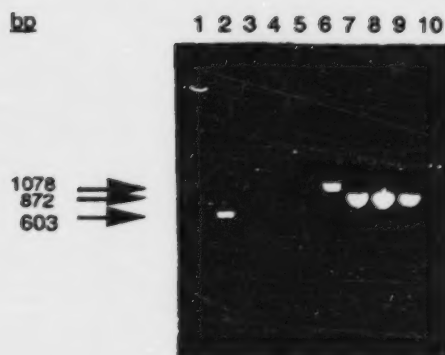


Figure 7. PCR Amplification of GFPuv

PCR amplification was carried out in 100 μ l reactions using 30 cycles of 94°C for 1 min., 54°C for 1 min., 72°C for 1 min. followed by 1 cycle of 72°C for 15 min. The products were then analyzed on a 1% agarose gel stained with ethidium bromide. The positive control was supplied with the GeneAmp PCR kit from Perkin Elmer (produces a 500 bp band). Negative controls for primers consisted of the appropriate primer pairs plus all components of the PCR reaction except template material. Template material for the PCR of GFPuv was either uncut pGFPuv, Eco RI digested pGFPuv, or LMPA purified GFPuv gene from Eco RI/Hind III digested pGFPuv. The GFPuv gene is 716 bp while our expected PCR product is 738 bp.

Each lane contains 10 μ l of the indicated sample:

Lane 1 = Lambda Hind III marker; Lane 2 = positive control supplied with the GeneAmp PCR kit from Perkin Elmer (produces a 500 bp band); Lane 3 = GFPuv 5' primer negative control; Lane 4 = GFP 3' primer negative control; Lane 5 = GFP 5'/GFP 3' primer negative control; Lane 6 = D12-3 PCR product; Lane 7 = PCR of uncut pGFPuv template; Lane 8 = PCR of Eco RI digested pGFPuv template; Lane 9 = PCR of Eco RI/Hind III digested pGFPuv template; Lane 10 = ϕ x174 marker.

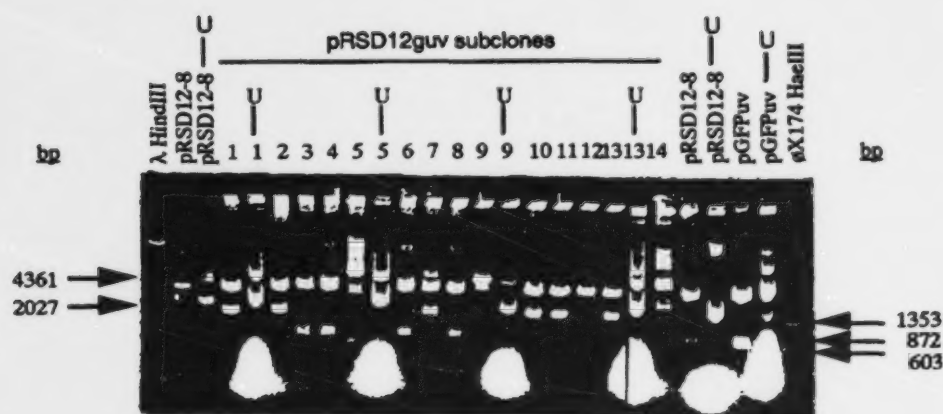


Figure 8. Screening RSD12guv Clones by Restriction Digestion.

The 738 bp GFPuv product was digested with Hind III and Not I and purified on a 1% LMPA gel. 0.2ug of purified product was ligated with 0.24ug of Hind III/Not I digested RSD12-8 and transformed into competent TG1 cells. 14 colonies were picked from the transformation plates and inoculated into 1ml of LB-AG. After 7 hrs. of growth, the cells were harvested and plasmid DNA was isolated. Recombinant plasmid was then digested with Eco RI/Hind III and the products were then analyzed on a 1% agarose gel stained with ethidium bromide. All samples were digested with Hind III and Eco RI unless specified (U = undigested plasmid).

digestion (cleaves 5,328 Da from the N-terminus). Expression is under the control of the T7 promoter.

Expression of GFPuv and RSD12guv were tested in various *E. coli* strains (TG1, DH1/pGP1-2, BL21, BL21(DE3), BL21(DE3) pLys S). Total cell lysates of induced clones in the above strains were analyzed by 15% SDS-PAGE and stained with coomassie brilliant blue. *E. coli* BL21 was shown to express the highest levels of GFPuv/scFv-GFPuv fusion product and was therefore chosen to be the expression host (data not shown). As shown in figure 9, plate cultures expressing functional GFPuv are readily detected by illuminating the plate with UV light. Broth cultures expressing RSD12guv failed to fluoresce when illuminated by UV light and therefore had to be detected by western blot analysis as shown on figure 10. When total cell lysates of induced *E. coli* BL21 transformed with pGFPuv (Clontech) were western blotted and immuno-stained with rabbit α -GFP pAb (Clontech), a 26-27kDa band corresponding to GFPuv was detected that was not present when the blot was immuno-stained with mouse α -express mAb (Invitrogen Corp.). When total cell lysates of induced *E. coli* BL21 transformed with pRSD12guv were western blotted and immuno-stained with rabbit α -GFP pAb (Clontech), a major band (60-61kDa) representing the scFv-GFPuv fusion product was observed. This band corresponds to 34kDa from RSD12 and 26.6kDa from GFPuv. Minor bands at 47kDa, 43kDa, and 27kDa were also present. These minor bands probably represent products generated from the proteolytic cleavage of the fusion product. The 27 kDa band represents GFPuv. When the same blot was immuno-stained with mouse α -express mAb (Invitrogen Corp.) major bands at 60-61kDa, 27 kDa and 21.5 kDa were observed. The 60-61kDa band represents RSD12guv. A minor band at 35kDa representing the scFv portion of the fusion product was also observed.

Purification of RSD12guv

RSD12guv is expressed as insoluble cytoplasmic inclusion bodies and was prepared for IMAC column purification as indicated in methods and materials. Material bound to the IMAC column was renatured by washing with a 6M-0M Gu-HCl step gradient. Functional activity was assayed by illuminating scFv-GFPuv fusion products with uv-light and looking for green fluorescence. IMAC-purification of RSD12guv-1 is shown in figure 11. When purified fractions were western blotted and immuno-stained with rabbit α -GFP pAb (Clontech), a 61kDa fusion product was observed. The same blot immuno-stained with mouse α -express mAb (Invitrogen Corp.) had bands at 61kDa (RSD12guv), 35kDa (RSD12), 33kDa, and 21.5kDa. No

fluorescence was detected from crude bacterial lysates or IMAC purified and renatured fractions indicating that the GFPuv moiety was incorrectly folded.

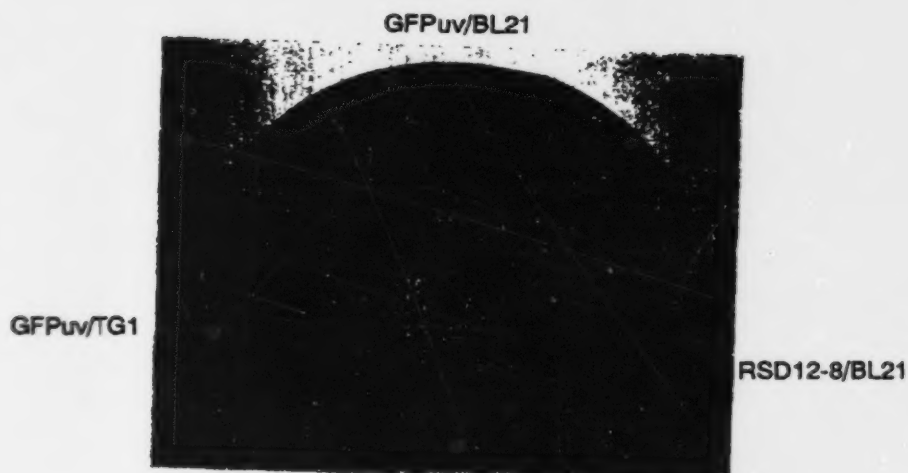
Identification of anti-ovalbumin monoclonal hybridomas to ovalbumin

Monoclonal hybridomas against ovalbumin were generated at Southern Alberta Cancer Research Center (SACRC). Six parental hybridomas secreting antibody reactive against ovalbumin were generated from the myeloma cell fusion and were further subcloned to yield twenty six mouse monoclonal hybridomas. Media supernatants from the twenty six hybridoma subclones were assayed by indirect ELISA using lysozyme (from chicken egg white), purified ovalbumin grade VI, chicken egg albumin, and BSA fraction V immobilized to 96-well plates. Immuno-complexes were undetectable using α -mouse-HRP conjugate and ABTS substrate. A triple layer detection method involving biotinylated α -mouse IgG, streptavidin-AP, and PNPP substrate was used to amplify the signal. As shown in Tables 2 and 3, all 26 hybridomas were reactive against ovalbumin, chicken egg albumin, and lysozyme. Previous data suggests that lysozyme binds non-specifically to many proteins, therefore the affinities of the hybridoma mAb's for lysozyme were attributed to the "sticky" nature of this protein. 1E11 hybridomas reacted with all of the test antigens on the plate indicating that these antibodies interact in a non-specific manner. 1D9, 8G1, 5G6, 5G7, and 12H6 hybridomas were specific to ovalbumin. The hybridoma 12H6H8F3 was chosen to be cloned as a scFv.

Cloning of anti-ovalbumin scFv 12H6H8F3

α -ovalbumin antibodies were generated using the recombinant phage antibody system (RPAS) (Pharmacia Biotech Canada). Total RNA from the mouse monoclonal hybridoma 12H6H8F3 (From SACRC) was reverse-transcribed and the VH and VL regions were PCR-amplified and joined by a (Gly₄Ser)₃ linker to yield a 758 bp assembled scFv with 5' Sfi I and 3' Not I sites (data not shown). 12H6H8F3 DNA was cloned into the Sfi I/Not I site of pCANTAB 5E and transformed into E. coli TG1 cells. Ten transformants were randomly picked and screened by restriction endonuclease digestion. All ten clones were identified as genetically positive. Nine of the ten clones contain full length scFv since they produced the expected 2112 bp and 3111 bp bands after Eco RI/Hind III digestion (Figure 6-B) and one clone contains a truncated scFv. Recombinant 12H6H8F3-M13 phage was rescued from each of the ten clones and applied to an PVC 96 well plate coated with ovalbumin. Upon development of the ELISA plate, all clones

Panel A: White light with no filter.



Panel B: UV illumination with coomassie filter.

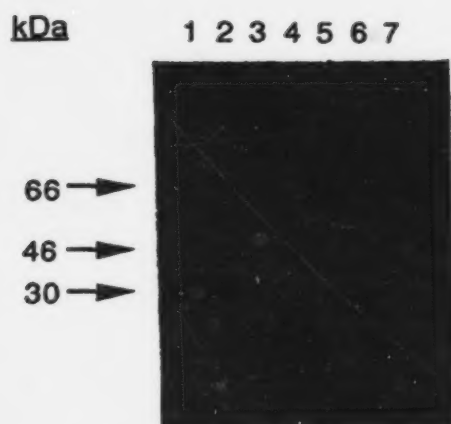


Panel C: UV illumination with modified coomassie filter.



Figure 9. Fluorescence of *E. coli* transformed with pGFPuv. pGFPuv and pRSD12-8 were transformed into *E. coli* BL21 or TG1. Single colonies were streaked onto an induction plate (LB agar plate supplemented with 100µg/µl ampicillin, 1mM IPTG) and grown over night at 30°C. Panel A depicts the fluorescence of GFPuv and RSD12-8 when the plate was illuminated with white light and a B/W (Black and White) photograph was taken with no filters. Panel B depicts the fluorescence of GFPuv and RSD12-8 when the plate is illuminated with UV light and a color photograph was taken with a coomassie filter. Panel C depicts the fluorescence of GFPuv and RSD12-8 when the plate was illuminated with UV light and a B/W photograph was taken with a modified coomassie filter set.

Panel A: Anti-GFP



Panel B: Anti-EK

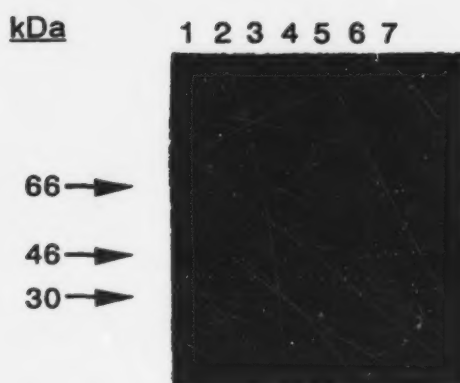


Figure 10. Expression of recombinant GFPuv (27 Kda) and D12-GFPuv fusion product (60.6 Kda) in *E.coli*. pGFPuv and pRSD12guv were transformed into *E. coli* BL21. Transformant subclones were grown overnight and bacterial cell lysates were prepared and separated by electrophoresis on 15% SDS PAG. Panel A was immunostained with rabbit α -GFP pAb/goat α -rabbit AP conjugate and developed with CSPD substrate. Panel B was immunostained with α -EK mAb/ donkey α -mouse HRP conjugate and developed with Supersignal CL chemiluminescent substrate (Pierce).

Each lane contains 8 ul of the indicated sample.

Lane 1 = BI21. Lane 2 = GFPuv. Lane 3 = RD12guv-1. Lane 4 = RD12guv-7. Lane 5 = RD12guv-10. Lane 6 = RD12guv-11. Lane 7 = BSA marker.

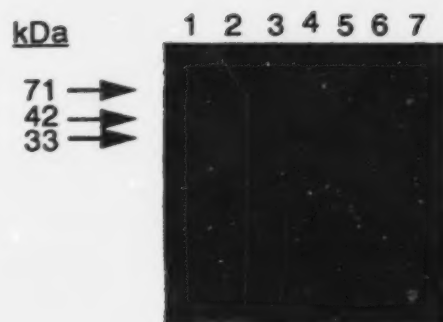
Figure 11. Expression of RSD12guv and purification by IMAC chromatography (1 of 2).

250ml culture of RSD12guv was grown and induced. Cell lysates prepared after induction, and fractions collected from the IMAC column were separated on a 15% SDS-PAG and transferred to a nitrocellulose membrane. Duplicate gels were run, one was stained with coomassie brilliant blue [Panel C] while the other was blotted onto nitrocellulose and probed with rabbit α -GFP(1/2000) (Clontech)/ goat α -rabbit-AP (1/2000) [Panel A]. Signals were detected with CSPD chemiluminescent substrate. The blot was stripped and probed with mouse α -express mAb(1/5000) (Invitrogen Corp.)/donkey α -mouse IgG-HRP(1/20,000) [Panel B]. Signals were detected with Supersignal CL Chemiluminescent substrate (Pierce).

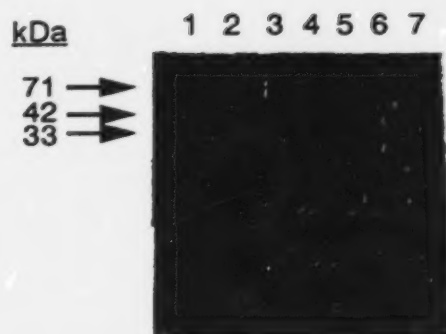
Each lane contains 15 ul of the indicated sample.

Lane 1 = crude lysate. Lane 2 = Column Flow Through. Lane 3 = last column wash. Lane 4 = Kaleidoscope prestained marker (BioRad). Lane 5 = Fraction 1. Lane 6 = Fraction 2. Lane 7 = BSA marker (2 μ g).

Panel A: α -GFP pAb Stain



Panel B: α -express mAb stain



Panel C: Commassie Blue stain

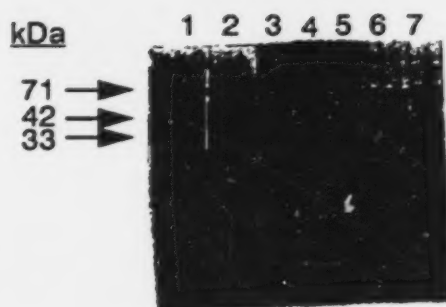


Figure 11. (2 of 2)

TABLE 2. SPECIFICITY OF MOUSE MONOCLONAL ANTIBODIES

Antibody	Lysozyme	Ovalbumin	Chick Egg		BSA	BLANK
			White			
mouse IgG 1/100	++	+	+		-	-
1E11E9E5	+++	+++	+++		++	++
1E11E9E11	+++	++	++		++	++
1E11F6E5	+++	+++	+++		+++	+++
1E11F6E6	+++	++	++		++	++
1E11F11E3	+++	+++	+++		+++	+++
1E11F11E4	+++	+++	+++		+++	+++
8G1D5E3	++	+++	++		-	-
8G1D5E7	++	+++	++		-	-
8G1D7E5	++	+++	++		-	-
8G1D7E7	++	+++	++		-	-
8G1E8E1	++	+++	++		-	-
8G1E8F10	++	+++	++		-	-
12H6E6F7	+++	+++	++		-	-
12H6H8F3	++	+++	++		-	-
BLANK	+++	-	-		-	-

Note: Data is compiled from 7 replicate ELISA plates. Each monoclonal antibody test sample was assayed in duplicate on each ELISA plate. ELISA staining scheme includes 1) test antibody 2) biotinylated goat anti-mouse IgG and 3) streptavidin-alkaline phosphatase conjugate and 4) nitrophenyl phosphate substrate

TABLE 3. SPECIFICITY OF MOUSE MONOCLONAL ANTIBODIES

Antibody	Chick Egg				BSA	BLANK
	Lysozyme	Ovalbumin	White			
5G7D9	++	+++	+++		-	-
1D9C12D6	++	+++	++1/2		-	-
5G7C3-1-F9	++	+++	+++		-	-
5G7C3-2-E2	++	+++	+++		-	-
5G7C3-2-F9	++	+++	+++		-	-
5G7C3-1-F5	++	+++	+++		-	-
5G7C32G3	++	+++	+++		-	-
5G6D9E9	++	+++	+++		-	-
5G7D9E9	++	+++	+++		-	-
5G7C3-2-E2F4	++	+++	+++		-	-
5G7C3-2-F9G6	++	+++	+++		-	-
5G7C3-2-F9G9	++	+++	+++		-	-
TEST ANTIBODY	+	+	+		+	+
BLANK	++	-	1/2+		-	-

Note: Data is compiled from 2 replicate ELISA plates. Each monoclonal antibody test sample was assayed in duplicate on each ELISA plate. ELISA staining scheme includes 1) test antibody 2) biotinylated goat anti-mouse IgG and 3) streptavidin-alkaline phosphatase conjugate and 4) nitrophenyl phosphate substrate

showed a slight reactivity towards ovalbumin (data not shown).

Discussion

The production of genetically engineered fluorescent antibodies would be advantageous in terms of purity, functional activity, and homogeneity of labelling. Previously we have labelled 3 Ω 7, a scFv against BoTN/A, and mouse α -ovalbumin IgG (Sigma) with Cy5 Dye (Amersham Life Science) for detection of Ab-Ag complexes by CE (unpublished data). CE analysis indicated that Cy5 labelled α -ovalbumin lost all binding activity and that the single peak corresponding to 3 Ω 7 scFv was resolved into a doublet after Cy5 labelling. Loss of binding activity is presumably due to labelling of the Ag binding site, while the generation of an additional peak indicates heterogeneity in labelling. A dye:protein ratio of 7-9 was calculated for both antibodies. The genetic tagging of antibodies with fluorescent molecules ensures that the antibody retains its biological activity and that there is exactly one fluorescent molecule/antigen binding site.

The bioluminescent photoproteins obelin and aequorin have been used as fluorescent tags (12-14). The fusion proteins retained both the luminescent activity of obelin/aequorin and the biological activity of the other fusion partner. Obelin (15) and aequorin (10) both require co-factors (Ca^{2+} /luciferin) in order to fluoresce and are therefore less suitable for tagging immunodetection reagents. GFP would be a superior fluorescent tag because its fluorescence does not require any co-factors (unless they are ubiquitous), is not species specific (16) and, a number of mutant GFP's have been generated with altered excitation/emission spectra (eg. UV excitation/blue emission, violet-blue excitation/cyan emission, cyan excitation/green emission) (17), solubility, rate of chromophore formation (18), fluorescence intensity (17, 19), and resistance to photobleaching (20). GFP mutants have been generated which are optimized for FACS/CE-LIF (eg. excited with argon laser (488nm) and detection with standard FITC filter set) (21) and expression of GFP in murine cells has been detected by FACS (22) while free GFP/GFP- α -GFP antibody complex were resolved within minutes and detected at the picogram level using CE-LIF (23).

The low level of fluorescence observed for the rGFP chemically coupled beads may be the result that GFP has lower fluorescence specificity or fluorescence quantum yield (ref). It would be important to measure equal molar amounts of GFP vs FITC. The low levels of fluorescence maybe attributed to low numbers of rGFP molecules/bead in addition to chemical inactivation by EDC. Broad Scatter profile may indicate possible crosslinking of beads. Regardless, GFP beads are detectable by FACS.

Simulation using PS beads are based upon the assumption that chemical coupling of

protein i.e. GFP or IgG to beads is highly efficient so that antigen will be displayed on 100% of the beads. It is more likely that chemical coupling of proteins to beads will be much less efficient and antigen binding-site inactivation by the chemical coupling is a real possibility. Inefficient display of active binding domains on beads may give rise to large proportion of beads that are not fluorescent in the FACS analysis. In contrast, in the actual detection of bacteria or spores, antigen binding sites or epitopes are displayed on 100% of the particular bacterial cells or spores. We would anticipate that detection signals using intact bacteria or spores would yield much more intense fluorescent signals in FACS spectrograms.

Success in producing biologically active fusion proteins requires that the joined molecules can fold independently to reconstitute active domains. A considerable number of functional GFP fusion proteins, both N- and C- terminal, have been constructed (16, 24, 25), and used to follow protein transport into cellular organelles (26), through the secretory pathway (27), to detect viral infected cells (28), and follow the developmental fate of cells (25). Our current expression system expresses scFv-GFP fusion products directly into the cytoplasm. The cytoplasm of *E. coli* is a reducing environment (29) and thus, the disulfide bond formation required for native conformation (30) of our fusion product does not occur. Although we obtain high levels of fusion product, the products are likely reduced, misfolded, form insoluble cytoplasmic inclusion bodies and must be refolded *in vitro*. Renatured RSD12guv failed to fluoresce when illuminated by UV light indicating that the domains of the fusion product did not attain a native conformation. The yields of functional protein from *in vitro* folding are often low, necessitating the removal of inactive protein. While functional antibody can readily be separated from inactive antibody by affinity chromatography, this can be problematic in large-scale production when the antigen of interest is difficult to obtain.

The scFv-GFP fusion product may form non-native conformations because the two domains are interacting with each other. When the scFv-GFP gene is transcribed, the scFv domain is translated first, followed by the GFP domain. Evidence suggests the scFv domain is folding improperly (unpublished data), which may be "guiding" the improper folding of the GFP domain. GFPuv is expressed as a soluble, fluorescent, extremely stable protein in *E. coli*, and therefore, the construction of a GFP-scFv vector (eg. switch domains in RSD12guv) could allow functional GFP-scFv fusion product to be expressed. Construction of pGuvD12, a GFPuv-scFv expression vector, has been initiated (Fig. 12). Expression in pGuvD12 is driven from the Lac Z promoter. The Lac Z promoter is weaker than the T7 promoter which will result in lower levels of recombinant protein being expressed. The lower level of expression may prevent the formation of inclusion bodies, typically observed with the T7 system, resulting in soluble, fluorescent scFv's.

It has been reported that a thiol-reducing agent is necessary for functional renaturation of GFP (37). GFP contains two cysteine residues in its secondary sequence which can participate in disulfide formation. The formation of GFP-GFP/scFv-GFP disulfide bonds would hinder the acquisition of native structure resulting in a non-fluorescent fusion protein. Transport into the periplasm of *E.coli* is functionally equivalent to transport into the endoplasmic reticulum of eukaryotic cells. The periplasmic space of *E.coli* is an oxidizing environment and possesses the enzymes and molecular chaperones necessary for proteins to obtain an active state. The genetic modification of our current vector to direct expression to the periplasm (eg. addition of Pel B signal sequence), could allow us to purify functional, scFv-GFP fusion products. Although yields from periplasmic expression are low, the ability to purify functional fusion product without the need for *in vitro* folding would be beneficial. Functional Fvs, scFvs, Fabs, and chimeric antibodies have been expressed in the periplasm of *E.coli* (31-36). In contrast to refolding cytoplasmically expressed proteins, *in vitro* folding of periplasmic recombinant proteins, in which correct disulfide bond formation has occurred, is often possible with high yields (30).

Chromophore formation of GFP has been proposed to proceed through a two-step process consisting of autocatalytic cyclization of the chromophore-forming tripeptide (⁶⁵Ser-Tyr-Gly⁶⁷) followed by oxidation (18). Molecular O₂ is sufficient for the oxidation step and thus adequate aeration during expression/renaturation is necessary. We have found that aeration generated by shaking a 10 ml culture in a 50 ml tube at 250 rpm may not be sufficient for native GFP to acquire fluorescence. While this is not the cause of lack of fluorescence of RSD12Guv, one must be aware that exposure of non-fluorescent, GFP fusion product to molecular O₂ may be sufficient to gain fluorescence.

Inspection of the primary sequence of pGFPuv and pD12 indicate numerous ribosomal binding sites (RBS) and sequences that can form stable hairpins. Four expression products are routinely detected: 1) 60-62 kDa scFv-GFP fusion product, 2) 30-35 kDa scFv product 3) 25-27 Kda GFP product, 4) 19-22 kDa product. The scFv and GFP products are minor products most likely resulting from proteolytic cleavage around the fusion site. Alternatively, these products could be formed by ribosomal bumping. Each of the fusion partners is preceded by a RBS (eg. N-terminus-RBS--6-His/EK-----D12-----RBS--GFP-----C-terminus). Full length fusion product is generated when a ribosome initiates translation at the N-terminal RBS and proceeds uninterrupted, straight through to the C-terminus. GFP would be generated by a ribosome initiating translation at the RBS just inside the GFPuv gene while scFv product would be generated by a ribosome beginning translation at the N-terminal RBS and being bumped off the mRNA transcript as another ribosome is binding to the RBS inside of GFP. The 19-22 kDa product can

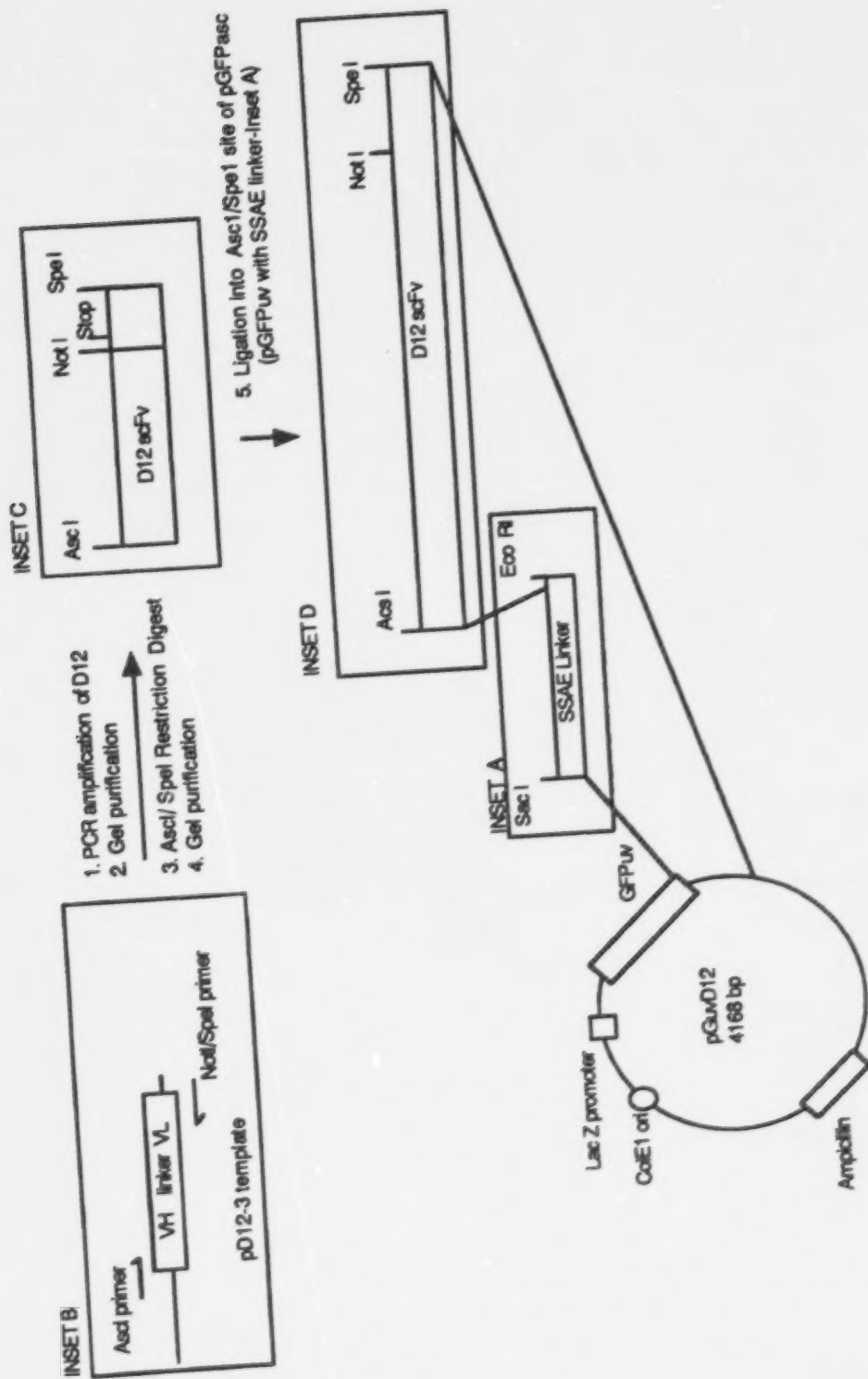


Figure 12. Construction of pGuvD12- a GFP fusion vector (1 of 4).
 A unique AscI site for cloning scFv's is introduced into pGFPuv via the SacI - SacII - AscI - EcoRI linker (SSAAE linker). This linker is PCR-amplified using AscI primer and NotI/SpeI primer. The purified PCR product was digested with AscI/SpeI and cloned into the AscI/SpeI sites of pGFPasc. The resulting vector will express scFv's as C-terminal fusion product with GFPuv.

Inset A: Cloning SSAE linker into Sac I/Eco RI digested pGFPuv.

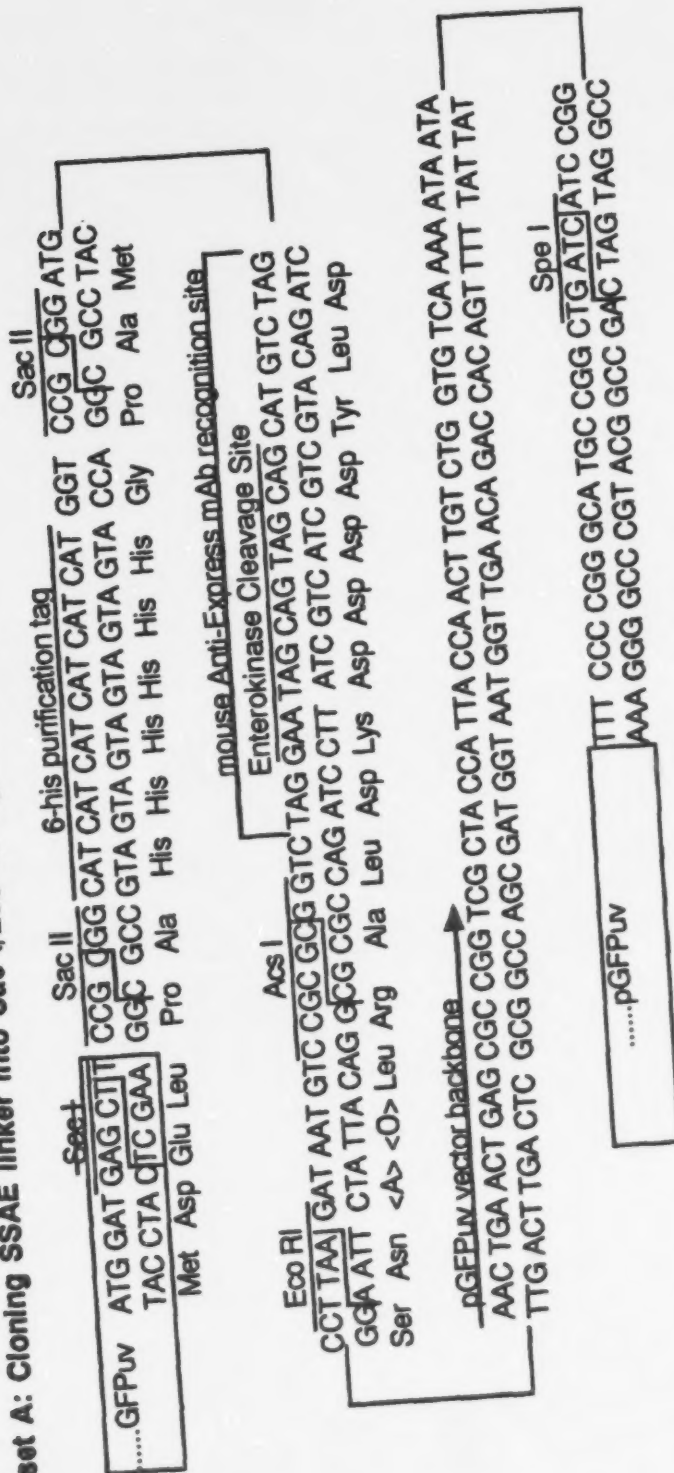
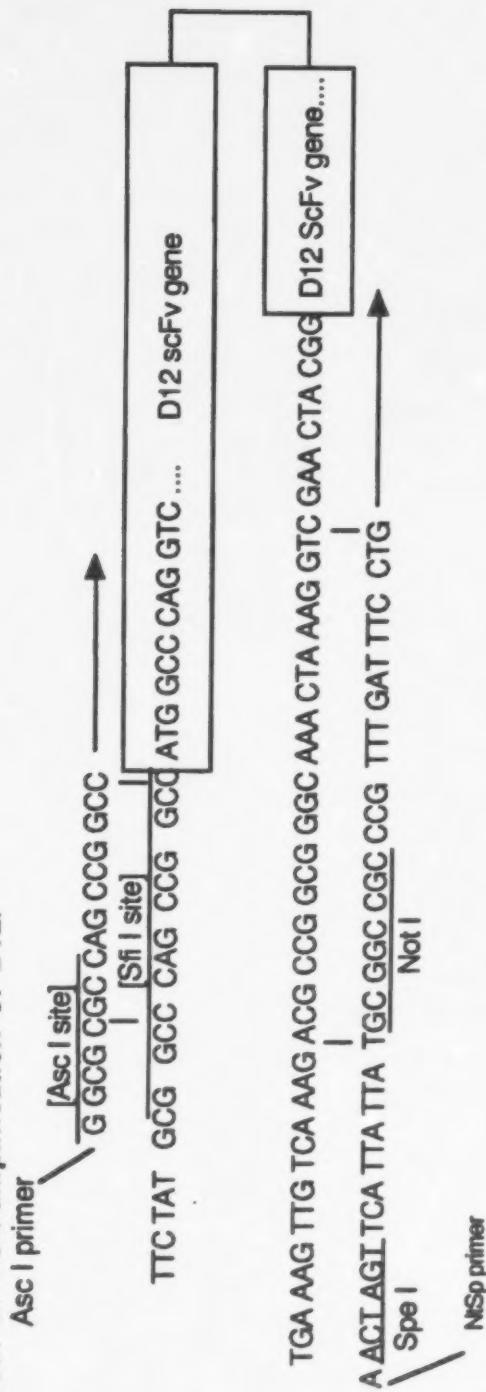
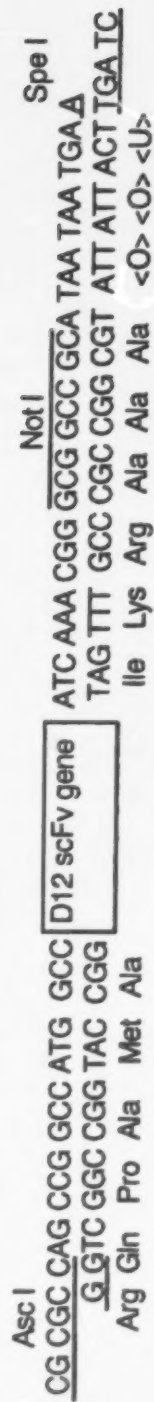


Figure 12 (2 of 4).

Inset B: PCR-amplification of D12.



Inset C: Asc I/Spe I Restriction Digestion of D12 scFv PCR product.



Inset D: Ligation of Asc I/Spe I digested D12 PCR product into Asc I/Spe I sites of pGFPasc.

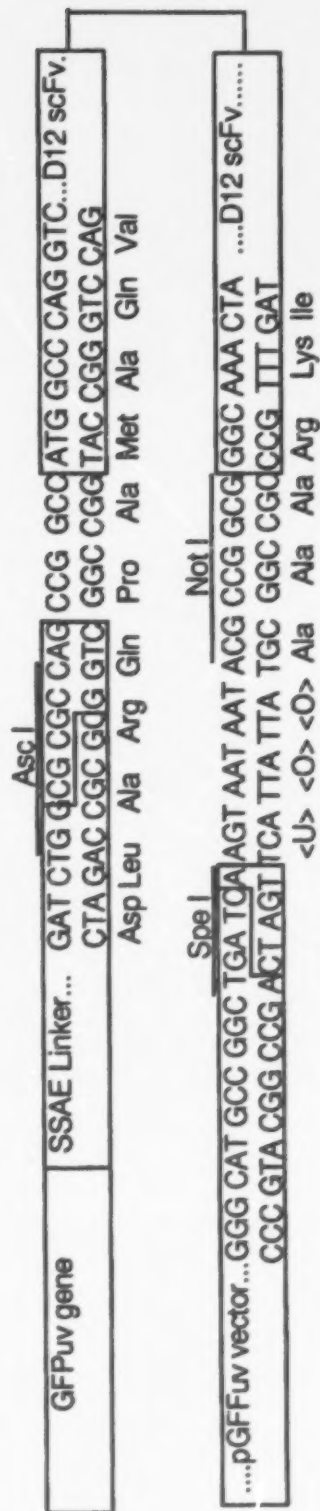


Figure 12 continued (4 of 4)

be a major or a minor product. It likely results from the formation of a stable hairpin structure in the linker peptide joining the V_H/V_L gene sequences. Alternatively it could be produced by proteolytic cleavage in the linker region.

Fv fragments, currently the smallest antibody fragments containing the complete antigen-binding site, are capable of antigen-combining and neutralization of biological activity. Comparable antigen-combining affinities for scFv's and other cloned antibody fragments with intact parental IgG have been obtained (34, 38, 39). The antigen-combining properties of the expressed fusion products are associated with the scFv domain. This region can be genetically modified to increase binding affinity. The V_H/V_L pairing in the α -ovalbumin scFv was selected naturally in mouse monoclonal hybridoma cell line 12H6H8F3. All intact α -ovalbumin monoclonal IgG's were undetectable in standard indirect antibody capture ELISA assays using ABTS substrate for colorimetric detection. A triple layer detection method involving biotinylated α -mouse IgG, streptavidin-AP, and PNPP substrate was needed to amplify the signal. ELISA's for detecting phage displayed antibody have not been adapted to this more sensitive technique and thus the weak signal observed in the ELISA using 12H6H8F3 phage antibody may reflect the weak binding properties of the parental IgG. Mutagenesis studies and research to improve antigen-combining properties [targeting of CDR regions] can be performed using genetic vectors harbouring the scFv gene only. scFv genes with optimized antigen-combining properties could then be cloned into the scFv-GFP fusion vector for expression and testing.

Disulfide stabilized Fvs(dsFvs) are antibody fragments in which the V_H and V_L chains are joined by interchain disulfide bonds (instead of a peptide linker as in scFv). dsFv's often have improved binding activity and a reduced tendency to aggregate, making them more stable than scFvs (40). The construction of dsFv's would allow us to produce a more stable fusion protein and could offer higher yields of purified protein (due to decreased aggregation during refolding/purification). The removal of the linker peptide joining the V_H and V_L would also remove potential RBSs and sequences thought to participate in hairpin formation from the mRNA. The removal of these sequences will result in less truncated product being expressed, allowing resources to be devoted to full length product.

Future Work

Future work should focus on three areas:

1) Generation of GFP fusion vector expressing fluorescent fusion products. Expression of RSD12guv in a T7 system results in the formation of insoluble, non-fluorescent inclusion bodies (presumably due to the high level of expression attained in T7 based expression systems). Four additional vectors should be constructed in attempts to express fluorescent fusion products. (1) Clone RSD12guv genetic construct into a vector in which expression is driven from the weaker Lac Z promoter. The lower expression levels may enable the expressed product to remain soluble. (2) Complete construction of pGuvD12. In addition to expression in pGuvD12 being under control of the Lac Z promoter, the dsFv/scFv is fused to the C-terminus of GFPuv. GFPuv expressed from the Lac Z promoter in *E. coli* remains soluble and fluorescent, thus the orientation of the fusion partners in GuvD12 should allow GFPuv to fold properly and perhaps "guide" the proper folding of dsFv/scFv domain. (3) The genetic modification of pRSD12guv/pGuvD12 to include a pel B leader sequence to direct expression to the periplasm. Secretion into the periplasm will allow proper disulfide bonds to form which should result in functional protein.

2) Genetic engineering of dsFv/scFv for advantageous mutations. Genes cloned into bacterial vectors, provide the genetic materials for mutagenesis (improving antigen-binding properties). Methods for producing genetically engineered mutants should be simple with rapid assays available to characterize the usefulness of the genetic mutations. The binding affinities of dsFvs/scFvs can be optimized by chain shuffling and CDR mutagenesis. Once optimal dsFvs/scFvs are obtained, they can be subcloned into the GFP fusion vector.

3) Genetic engineering of GFP for advantageous mutations. Existing GFP mutants with altered excitation/emission spectra and increased fluorescence intensity can be cloned and used as the fusion partner instead of GFPuv. Alternatively, DNA shuffling can be used to generate new GFP mutants.

Methods and Materials

Chemically coupling rGFP to polystyrene beads.

Recombinant GFP (Clontech) was coupled to carboxyl-polystyrene beads (3.54 μ diameter -Spherotech Cat.#CP-30-10) according to the manufacturers instructions. One hundred microliters (100 μ l) of 1 mg/ml rGFP was mixed with 100 μ l of 5% PS beads(polystyrene beads) and 10 μ l of 0.5 M MES pH 5.0. This mixture (210 μ l total volume) was transferred to a glass tube containing 10 mg EDC (1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Methiodide -Sigma Chemical Co.), mixed and allowed to incubate at ambient temperature for two hours. Beads were collected by centrifugation and inspected under UV-irradiation. Reaction supernatant was collected for SDS PAG and western blot analysis for the presence of uncoupled rGFP.

Immunocomplexing of rGFP to PS Beads.

Dilutions (1/20 and 1/200) of 1 mg/ml rGFP (Clontech) and Rabbit anti-GFP antisera were prepared in PBS pH 7.4. Fifty microliter (50 μ l) aliquots of different dilutions of rGFP, 50 μ l of different dilutions of rabbit anti-GFP antisera and 50 μ l of 0.25% goat anti-rabbit IgG coated PS beads (0.7 to 0.9 μ diameter -Spherotech Cat.#RPFc-08-20) were combined in a microfuge tube and incubated for 30 minutes. Immunocomplex-beads (GFP-IgG coated PS beads) were washed once with PBS prior to analysis by FACS.

FACS Analysis of GFP-beads and GFP-IgG coated beads

Dilutions (1/100 or 1/1000) of 2.5% GFP-beads or dilutions of (1/100 or 1/1000) of 0.083% GFP-IgG coated PS beads were analyzed using the Skatron Argus 1.72 FACS apparatus. After instrument calibration with standard fluorescent beads [B1 (1 μ diameter) beads, or Fluorobeads (FITC-10 μ diameter) beads], samples were processed for 1 minute intervals and the FACS spectrograms were printed out on plotter. Typically filters B1 and B2 were used in the running of the instrument.

Construction of the bacterial expression vector pRSD12guv.

The cloning strategy for the construction of pRSD12guv (a pRSET A T7- based expression vector expressing the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* fused to the carboxyl terminus of the scFv) is shown in figure 4.

PCR Amplification of GFPuv (Clontech).

2 μ g of pGFPuv was digested either with Eco RI or Eco RI/Hind III and the bands containing

GFPuv coding region (3337 bp and 778 bp respectively) were trapped in 0.8% low melting point agarose (LMPA). 20ng of supercoiled pGFPuv and 20ng of each of the above trapped bands were PCR amplified in separate 100µl reactions using GFP 5' Not and GFP 3' Hind primers (Appendix C) with 2.5 U Taq DNA polymerase. PCR amplification was carried out using 30 cycles of 94°C/1 min., 54°C/1 min., 72°C/1 min. followed by 1 cycle of 72°C/15 min. Amplified GFPuv containing 5' Not I and 3' Hind III restriction sites (738bp) was purified by LMPA and then ethanol precipitated.

Cloning GFPuv coding region into pRSD12-8

2µg of PCR amplified GFPuv and 1.1µg of pRSD12-8 were digested for 1 hr at 37°C with 10 units of Hind III in a 20µl reaction. The NaCl concentration was adjusted to 100mM and 15 units of Not I were added to the resulting digest and left for 1 hr at 37°C. Following digestion, the samples were purified by LMPA and 0.2µg of digested, purified, GFPuv (727 bp) DNA was ligated at 16°C for 1 hr with 0.24µg of digested, purified, RSD12-8 (2940 bp band) in a 10µl ligation reaction. 0.13µg of ligated DNA was transformed into competent *E.coli* TG1 cells. pRSD12guv transformants were inoculated into 1 ml LBA broth (LB broth supplemented with 200µg/µl ampicillin) and recombinant plasmid isolated by the alkaline-lysis method. 0.2µg of plasmid DNA was digested with 10 U Hind III and Eco R I in a 20µl reaction for 30 min. at 37°C and the resulting products were analyzed on a 1% agarose gel stained with ethidium bromide.

Expression and immuno-detection of RSD12guv Clones

pRSD12guv (from genetically positive clones) and pGFPuv were transformed (40-70ng DNA) into TG1 or BL21. Transformants were grown in 2ml of LBA broth at 37°C until OD₆₀₀ = 0.5-0.6. 1ml of each culture was removed and induced with IPTG (1mM final concentration). The cultures (induced and uninduced) were grown o/n at 30°C. The cells from each 1ml aliquot were lysed and analyzed by 15% SDS - PAGE. The gel was blotted onto nitrocellulose and probed for the presence of RSD12guv. The primary antibody in the immunostain was either mouse α-express antibody (Invitrogen Corp.) (reactive against the enterokinase cleavage site in all pRSET A recombinant proteins) or rabbit α- GFP (clontech). The secondary antibody was doukey α-mouse-HRP or goat α-rabbit-AP. Signals were detected with LumiGlo chemiluminescent substrate.

Generation of anti-ovalbumin mouse monoclonal hybridomas

Monoclonal antibodies were prepared by Southern Alberta Cancer Research Center (SACRC). Balb/C mice were vaccinated with 10 μ g of chicken egg albumin (Sigma, catalog #: A5253) followed by three injections of 10 μ g of purified ovalbumin grade VI (Sigma, catalog #: A2512).

Cell fusions were performed using standard SACRC hybridoma procedures. Serum titers of α -ovalbumin antibodies were assayed by ELISA and mice with titers > 10,000 were sacrificed for preparation of hybridomas.

Specificity of mAb's generated by SACRC

The following antigens were used to determine the specificity of the mAb's generated by SACRC: lysozyme (from chicken egg white), purified ovalbumin grade VI, chicken egg albumin, and BSA fraction V. The controls for this assay were mouse IgG and PBS. ELISA's were conducted as follows on Nunc Polystyrene 96 well flat bottom plates in duplicate assay format. 50 μ l of 2 x coating buffer (0.05 M Carbonate Buffer) and 50 μ l of 10 μ g/ml of the appropriate antigen/control were added to the wells and the plate was incubated overnight at 4°C. The wells washed 6 X's with PBST (0.1% Tween 20, PBS pH7.0) and blocked with 100 μ l blocking buffer (2% BSA, 0.1% Tween 20, PBS pH7.0) for one 1 hr at room temperature. The plate was washed as above and 100 μ l of culture supernatant from each hybridoma was added to the test antigens and incubated for 1 hr at 37°C. The plate was washed as above and 100 μ l of biotinylated goat α -mouse (1/500) was added to each well and incubated for 1 hr at 37°C. The plate was washed again and 100 μ l of streptavidin-AP (1/1000) was added to each well and incubated for 1 hr at 37°C. The plate was washed as above and 100 μ l of 4mg/ml solution of p-nitrophenyl phosphate in substrate buffer (100mM diethanolamine, 1mM MgCl₂, pH 10.0) was added to each well. The plate was kept in the dark for 30 min at room temperature before the plate was scored.

Cloning recombinant scFv antibodies reactive against ovalbumin

Anti-ovalbumin scFv antibodies were generated using the recombinant phage antibody system (RPAS)(Pharmacia Biotech Canada).

First-strand cDNA Synthesis of 12H6H8F3

Total RNA from the hybridoma 12H6H8F3 (Southern Alberta Cancer Research Center) was ethanol-precipitated and resuspended in DEPC-treated water to a final concentration of 2 μ g/ μ l. A 33 μ l reaction containing 10 μ g of RNA was reverse transcribed using SuperScript II from Gibco

BRL or as outlined in the RPAS kit (Pharmacia Biotech).

PCR Amplification of VH and VL DNA Fragments

The above first-strand reactions were used as template material to amplify the variable light (VL) and variable heavy (VH) DNA fragments. To the above tubes, a primer mix for the light chain or heavy chain was added (primer mixes supplied in RPAS kit) and 100 μ l PCR reactions were set up. There were 30 cycles of 94°C /1 min., 55°C/2 min., 72°C/2min. The products from these reactions were analyzed on a 1.5% agarose gel stained with ethidium bromide. The heavy chain (340 bp) and light chain (325 bp) fragments were excised from the gel and purified using empty MicroSpin Columns (supplied in the RPAS kit).

PCR Amplification of 12H6H8F3 scFv DNA

The purified VH and VL DNA fragments were linked together by a (Gly₄Ser)₃ linker peptide by combining 50ng VH and VL DNA, and 4 μ l of Linker Primer mix (RPAS kit) in a 50 μ l PCR/assembly reaction. There were 7 cycles of 94°C/1min., 63°C/4 min.. The assembled scFv was PCR amplified by combining the 50 μ l assembly reaction and 4 μ l RS Primer mix (RPAS) into a 100 μ l (final volume) PCR reaction with 30 cycles of 94°C/1 min., 55°C/2 min., 72°C/2min.. 12H6H8F3 scFv (750 bp) fragment was purified by spinning the PRC reaction through a MicroSpin Column containing 750 μ l of Sephacryl[®] S-400 HR resin at 735 X g for 2 min.

Cloning 12H6H8F3 scFv into pCANTAB 5E

350ng of assembled scFv was digested at 50°C for 4 hr. with 20 U of Sfi I in a 85 μ l reaction overlayed with 85 μ l mineral oil. 40 U of Not I were added to the resulting digest and the volume adjusted to 100 μ l with the appropriate buffer and digested at 37°C for 4 hrs. The digested sample was phenol:chloroform extracted and purified by spinning the extracted product through a MicroSpin Column containing 750 μ l of Sephacryl[®] S-400 HR resin at 735 X g for 2 min. 25ng of digested, purified, 12H6H8F3 scFv DNA was ligated at 16 for 1 hr with 50ng of Sfi I/Not I digested pCANTAB 5E in a 10 μ l ligation reaction. 30ng of ligated DNA was transformed into competent *E. coli* TG1 cells and single colonies were screened with Eco RI/Hind III restriction digests as described above.

Rescue of Recombinant 12H6H8F3 M13 Phage

Phage from positive r12H6H8F3 clones were rescued for ELISA analysis as follows: 400 μ l from overnight cultures of r12H6H8F3 clones were inoculated into 2ml of 2xYT containing 200 μ g/ml

ampicillin, 2% glucose, and 3×10^{10} pfu/ml M13K07. The culture was incubated at 37°C with shaking at 150 rpm for 2 hrs. The cells were pelleted by centrifugation for 10 min at 900 X g and the supernatant discarded. The cell pellet was resuspended in 5ml 2xYT containing 200µg/ml ampicillin, 50µg/ml kanamycin and incubated overnight at 37°C with shaking at 250 rpm. The cells were pelleted and the supernatant containing the recombinant phage was collected.

ELISA using Recombinant 12H6H8F3 M13 Phage

ELISA's were conducted as follows on Falcon PVC 96 well round bottom plates in triplicate assay format allowing 14 clones/plate to be analyzed. The wells around the perimeter of the plate were not used in the assay. The remaining 60 wells were divided into three identical assays. 50µl of 2 x coating buffer (0.1M sodium carbonate pH 9.2) was added to each of the assay wells. 50µl of 10µg/ml antigen (ovalbumin) was added to 15 of the wells/assay. The remaining 5 wells/assay received one of the following: 50µl 1x PBS, 50µl 10µg/ml BSA, 50µl of 10µg/ml lysozyme, 50µl 2µg/ml positive control antigen (Pharmacia sheep anti-M13), or 50µl positive control phage (Pharmacia M13K07 bacteriophage $>5 \times 10^{10}$ pfu/ml). The plate was then incubated overnight at 4°C. 150µl of rescued recombinant 12H6H8F3 phage and 30µl of blocking buffer (5% skim milk) were added per well (once for each assay). M13K107 helper phage and phage displaying a scFv against Botulinum toxoid (W925-E14 and W1002-52) were also tested in each assay. 150µl PBS was added to perimeter wells. Bound phage displaying scFv antibodies were detected by incubation with 100µl of 1:5000 sheep α-M13 HRP and subsequent color development with 90µl of ABTS substrate/well.

IMAC purification of RSD12guv

RSD12guv is expressed with a N-terminal leader peptide containing a 6-His tag for immobilized metal affinity chromatography (IMAC), the epitope for mouse α-express monoclonal IgG antibody (Asp-Leu-Tyr-Asp-Asp-Asp-Lys), and an enterokinase recognition site (Asp-Asp-Asp-Asp-Lys - cleavage occurs on the C-terminal side of Lys).

pRSD12guv (in BL21) was inoculated into 20ml of LBA broth and incubated at 37°C o/n. The o/n culture was inoculated into 250ml of LBA broth and incubated at 37°C until $OD_{600} = 0.5-0.6$. Expression of RSD12guv was induced by the addition of IPTG (1mM final concentration) and proceeded at 37°C for 3 hrs. The cell pellet was resuspended in 12ml of lysis buffer (4M urea, PBS pH 7.0) and sonicated for 60 seconds (6 X 10 sec. pulses). Insoluble material was pelleted by centrifugation at 15,000 X g for 15 min. and resuspended in 12ml of IMAC column buffer (6M

Gu-HCL, 150mM NaCl, 25mM borate buffer pH 9.0). Insoluble material was pelleted by centrifugation at 15,000 X g for 15 min. and the supernatant applied to a 1ml column of TALON Metal Affinity Resin (Clontech) equilibrated with IMAC column buffer. The column was washed with a Gu-HCl step gradient (2mls/step). The steps were 6M, 3M, 1.5M and 0.75M Gu-HCl in column wash buffer (25mM borate buffer pH 9.0, 150mM NaCl). After the final Gu-HCl step, the column was washed with 5ml of column buffer. Bound protein was eluted with elution buffer (25mM borate buffer pH 9.0, 100mM imidazole) in 4 X 2ml fractions. 3 μ l samples at each stage of purification were analyzed by 15% SDS-PAGE. Duplicate gels were run, one was stained with coomassie brilliant blue while the other was blotted onto nitrocellulose and probed with rabbit α -GFP(1/2000) (Clontech)/ goat α -rabbit-AP (1/2000). Signals were detected with CSPD chemiluminescent substrate. The blot was stripped and probed with mouse α -express mAb(1/5000) (Invitrogen Corp.)/donkey α -mouse IgG-HRP(1/20,000). Signals were detected with Supersignal CL Chemiluminescent substrate (Pierce).

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Appendix A: Calculation of Ratio of rGFP to PS Beads for Chemical Coupling.

100 ug of rGFP was reacted with 5 mg of PS beads

rGFP (MW = 27Kdal) or 27Kg/mole

$$\begin{aligned} 100 \text{ ug of rGFP} &= 3.7 \text{ nM} \times 6.023 \times 10^{14} \text{ molecules/nMole} \\ &= 1.27 \times 10^{10} \text{ molecules} \end{aligned}$$

3.54 um PS beads

$$\begin{aligned} \text{Vol. of sphere} &= \frac{4}{3} \pi r^3 \\ &= 23.2 \text{ um}^3/\text{bead} \end{aligned}$$

$$\begin{aligned} \frac{5 \text{ mg beads}}{1050 \text{ mg/cm}^3} &= \frac{4.76 \times 10^{-3} \text{ cm}^3 \times 10^{12} \text{ um}^3}{1 \text{ cm}^3} \\ &= 4.76 \times 10^9 \text{ um}^3 \end{aligned}$$

$$\begin{aligned} \text{No. particles} &= \frac{4.76 \times 10^9 \text{ um}^3}{23.2 \text{ um}^3/\text{bead}} \\ &= 2.05 \times 10^8 \text{ particles} \end{aligned}$$

$$\text{Therefore the ratio of GFP molecule/PS bead} = \frac{476 \times 10^7}{20.5 \times 10^7}$$

$$= 23 \text{ rGFP molecules/bead.}$$

By visualization of fluorescence of rGFP in reacted supernatant and reacted beads an estimate of 70 to 90% coupling efficiency.

Thus 16-21 molecules of GFP/bead.

Western blot analysis 10/200 0.5 ug <10ug in supernatant

VECTOR INFORMATION

pGFPuv Bacterial Vector

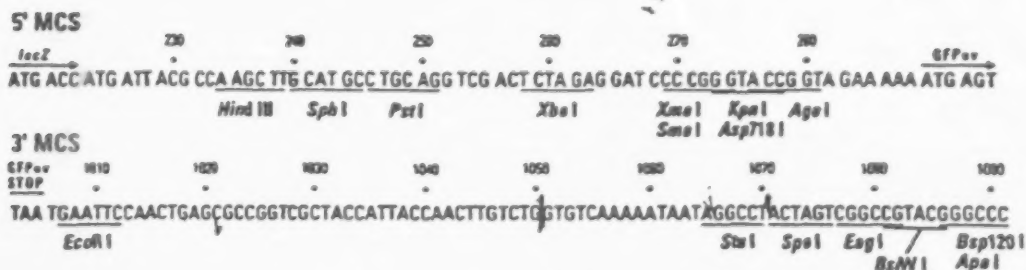
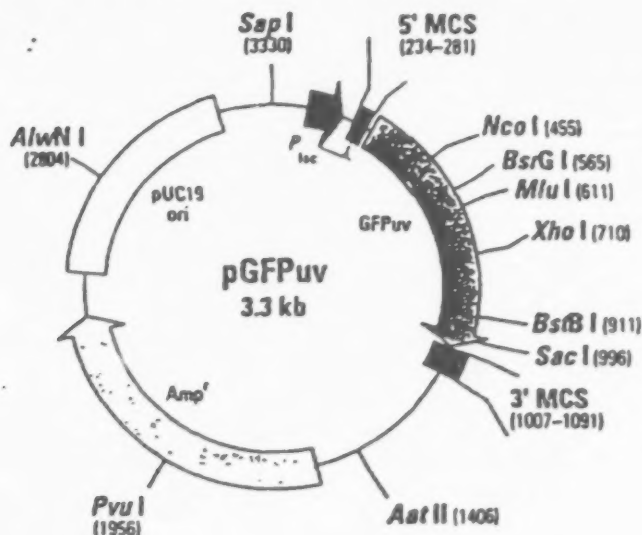
GenBank Accession #U62636

Appendix B.

Contract W7702-6-R600/001/EDM

PT3055-5

Catalog # 6079-1



Restriction Map and Multiple Cloning Sites (MCS) of pGFPuv. (Unique restriction sites are in bold.)

Description:

pGFPuv carries the "cycle 3" variant of GFP described by Cramer *et al.* (1). This gene was cloned between the two MCS of the pUC19 derivative pPD16.43 (2). The GFPuv coding sequence is flanked by separate MCS at the 5' and 3' ends, so the GFPuv gene can be easily excised from pGFPuv. Alternatively, the GFPuv coding sequence can be amplified by PCR. The GFPuv gene was inserted in frame with the *lacZ* initiation codon from pUC19 so that a β -galactosidase-GFPuv fusion protein is expressed from the *lac* promoter in *E. coli*. Note, however, that if you excise the GFPuv coding sequence using a restriction site in the 5' MCS, the resulting fragment will encode the native (i.e., non-fusion) GFPuv protein. The pUC19 backbone of pGFPuv provides a high copy number origin of replication and ampicillin resistance gene for propagation in *E. coli*.

(PR67194)

Location of Features:

- *lac* promoter: 95–178
 - CAP binding site: 111–124
 - 35 region: 143–148; –10 region: 167–172
 - Transcription start point: 179
 - lac* operator: 179–199
- *lacZ*–GFPuv fusion protein expressed in *E. coli*
 - Ribosome binding site: 206–209
 - Start codon (ATG): 217–219; Stop codon: 1003–1005
- 5' MCS: 234–281
- GFPuv gene
 - Start codon (ATG): 289–291; Stop codon: 1003–1005
 - GFP chromophore: 481–489
 - wt GFP cDNA sequences (3): 289–454
 - Synthetic GFP gene with "cycle 3" mutations from pBAD-GFPuv (1): 455–1007
 - Cycle 3 mutation F99S (T→C): 584
 - Cycle 3 mutation M153T (T→C): 746
 - Cycle 3 mutation V163A (T→C): 776
 - Cycle 3 silent mutation in L137 (T→C): 699
 - Cycle 3 silent mutation in T225 (A→T): 963
 - Q80R mutation (A→G) (4): 527
 - Arg codons optimized for *E. coli*: R73 (AgA→CgT): 505–507, R96 (AgA→CgC): 574–576, R122 (AgA→CgT): 652–654, R168 (AgA→CgC): 790–792, R215 (AgA→CgT): 931–933
 - Silent mutations (CccA→TccG) creating *Bsp*E I site: 510 & 513
 - Silent mutation (A→G) creating *Mlu* I site: 612
 - Silent mutations (TtGgaA→CtCgaG) creating *Xho* I site: 709, 711 & 714
 - Silent mutations (AG→TC) creating *Bam*H I site: 811–812
 - Silent mutation (C→G) creating *Sal* I site: 894
 - Silent mutations (ActA→GctC) creating *Sac* I site: 993 & 996
 - Silent mutation in S72 (A→C): 504
- 3' MCS: 1007–1091
- Ampicillin resistance gene
 - Promoter: –35 region: 1467–1472; –10 region: 1490–1495
 - Transcription start point: 1502
 - Ribosome binding site: 1525–1529
 - β -lactamase coding sequences:
 - Start codon (ATG): 1537–1539; Stop codon: 2395–2397
 - β -lactamase signal peptide: 1537–1605
 - β -lactamase mature protein: 1606–2394
- pUC plasmid replication origin: 2545–3188

Primer Location:

- GFP-N Sequencing Primer (#6476-1): 331–352
(Note: The GFP-C Sequencing Primer cannot be used with pGFPuv.)

Propagation in *E. coli*

- Recommended host strain: JM109 or DH5 α
- Selectable marker: plasmid confers resistance to ampicillin (100 μ g/ml) to *E. coli* hosts
- *E. coli* replication origin: pUC
- Copy number: ~500
- Plasmid incompatibility group: pMB1/ColE1

References:

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Appendix B.

Contract W7702-6-R600/001/EDM

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TCGCGGGTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAA
10 20 30 40 50 60 70

AseI PvuII
| |

TCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAG
AGGGCTGACCTTTTCGCCCCGTCACCTCGCGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTC
80 90 100 110 120 130 140

AseI
|

GCTTTACACTTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTACACAGGAA
CGAAATGTGAAATACGAAGCCGAGCATACAACACACCTTAACACTCGCCTATTGTTAAAGTGTGTCTTT
150 160 170 180 190 200 210

BsrBI
|

ACAGCTATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTGCGACTCTAGAGGATCCCCGGGTACCGG
TGTCGATACTGGTACTAATGCGGTTCCGAACGTACGGACGTCCAGCTGAGATCTCCTAGGGGCCCATGGCC
220 230 240 250 260 270 280

Sal I Asp718 I
| |

BspMI PstI XbaI XmaI KpnI
| | | | |

HindIII SphI Sse8387 I BamHI SmaI AgeI
| | | | |

TAGAAAAAATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTGAATTAGATGGTGA
ATCTTTTACTCATTTCCTCTCTTGAAAAGTGACCTCAACAGGGTTAAGAACAATTAACTTACCACT
290 300 310 320 330 340 350

TGTTAATGGGCACAAATTTTCTGTCACTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTT
ACAATTACCCGTGTTTAAAGACAGTCACTCTCCCACTTCCACTACGTTGTATGCCTTTTGAATGGGAA
360 370 380 390 400 410 420

NcoI MscI
| |

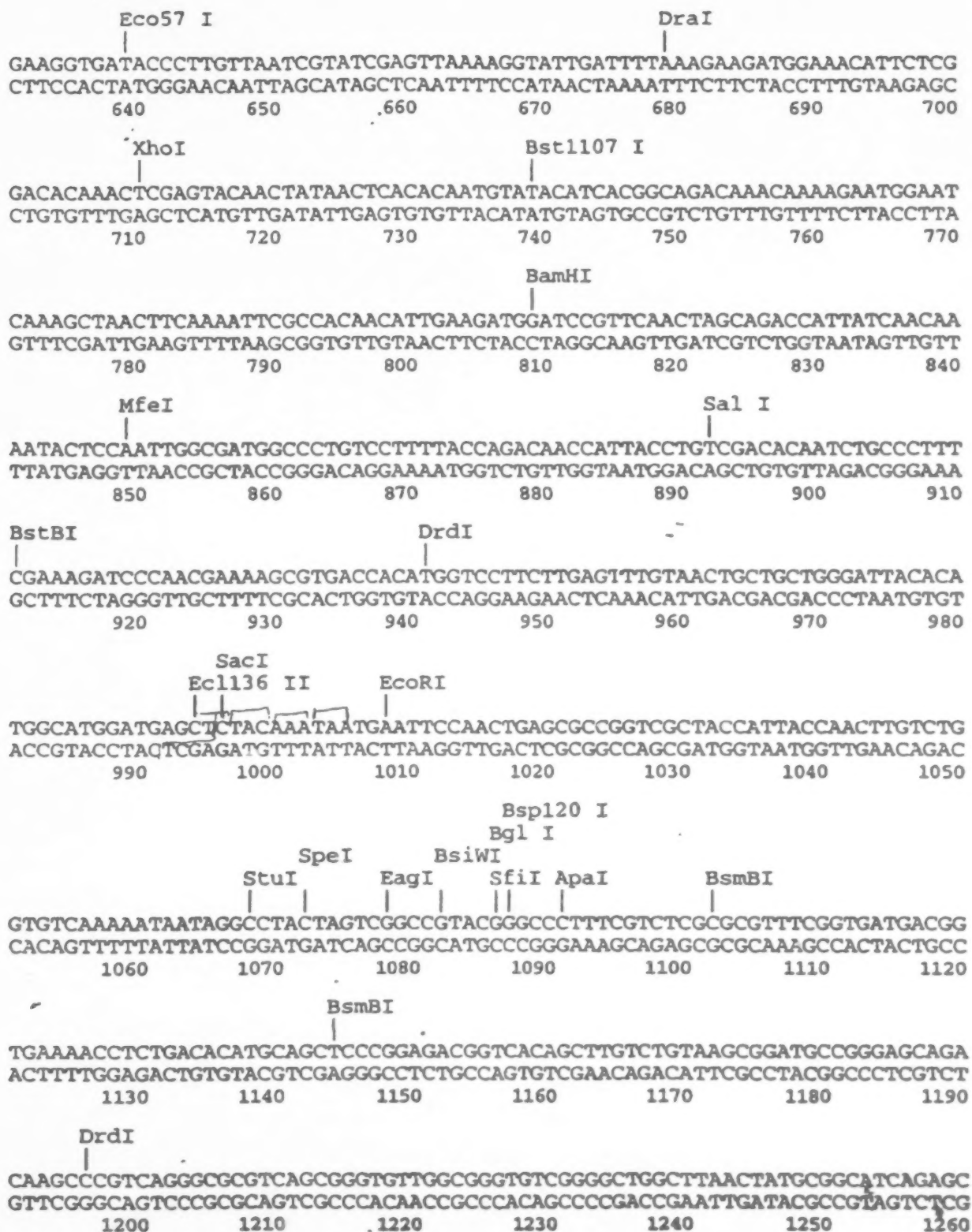
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TTTAAATAAACGTGATGACCTTTTGATGGACAAGGTACCGGTTGTGAACAGTGATGAAAGAGAATACCAC
430 440 450 460 470 480 490

BspEI NdeI
| |

TTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTCAAGAGTGCCATGCCCGAAGG
AAGTTACGAAAAGGGCAATAGGCCTAGTATACCTTTCGCGTACTGAAAAAGTTCTCACGGTACGGGCTTCC
500 510 520 530 540 550 560

BsrGI MluI
| |

TTATGTACAGGAACGCACTATATCTTTCAAGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTT
AATACATGTCTTGGCGTATATAGAAAGTTTCTACTGCCCTTGATGTTCTGCGCACGACTTCAGTTCAA
570 580 590 600 610 620 630



ApaLI NdeI
 | |
AGATTGTAAGTGCAGAGTGACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCAT
TCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTGTCTACGCAATCCTCTTTTATGGCGTA
 1270 1280 1290 1300 1310 1320 1330

 Bgl I
 SfiI
 | | | BspHI
 | | | |
CAGGCGGCCTTAAGGGCCTCGTGATACGCCTATTTTTATAGGTAAATGTCATGATAATTAATGGTTTCTTA
GTCCGCCGGAATTCCCGGAGCACTATGCGGATAAAAAATATCCAATTACAGTACTATTATTACCAAAGAAT
 1340 1350 1360 1370 1380 1390 1400

 AatII
 |
GACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCA
CTGCAGTCCACCGTGAAAAGCCCCCTTTACACGCGCCTTGGGGATAAACAATAAAAAGATTTATGTAAGT
 1410 1420 1430 1440 1450 1460 1470

 BspHI
 BsrBI SspI EarI
 | | | |
AATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGA
TTATACATAGGCGAGTACTCTGTTATTGGGACTATTTACGAAGTTATTATAACTTTTTCTTCTCATACT
 1480 1490 1500 1510 1520 1530 1540

GTATTCAACATTTCCGTGTCGCCCTTATTCCTTTTTTTCGGGCATTTTGCTTCCTGTTTTTGCTCACCC
CATAAGTTGTAAAGGCACAGCGGAATAAGGGAAAAACGCCGTAAAACGGAAGGACAAAAACGAGTGGG
 1550 1560 1570 1580 1590 1600 1610

 BssSI
 | ApaLI Eco57 I
 | | |
AGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGAT
TCTTTGCGACCACCTTTCATTTTCTACGACTTCTAGTCAACCCACGTGCTCACCAATGTAGCTTGACCTA
 1620 1630 1640 1650 1660 1670 1680

 XmnI
 | Psp1406 I DraI
 | | |
CTCAACAGCGGTAAGATCCTTGAGAGTTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAG
GAGTTGTCGCCATTCTAGGAACCTCTCAAAAGCGGGGCTTCTTGCAAAAGGTTACTACTCGTGAAAATTC
 1690 1700 1710 1720 1730 1740 1750

 BcgI
 |
TTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCCGCATACACTA
AAGACGATACACCGCGCCATAATAGGGCATAACTGCGGCCCCGTCTCGTTGAGCCAGCGGCGTATGTGAT
 1760 1770 1780 1790 1800 1810 1820

 BcgI ScaI
 | |
TTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGA
AAGAGTCTTACTGAACCAACTCATGAGTGGTCAGTGTCTTTTCGTAGAATGCCTACCGTACTGTCAATTCT
 1830 1840 1850 1860 1870 1880 1890

PvuI
|
GAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAG
CTTAATACGTCACGACGGTATTGGTACTCACTATTGTGACGCCGGTTGAATGAAGACTGTTGCTAGCCTC
1900 1910 1920 1930 1940 1950 1960

GACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACCTGCCTTGATCGTTGGGAACC
CTGGCTTCCTCGATTGGCGAAAAACGTGTTGTACCCCTAGTACATTGAGCGGAAGTACCAACCCCTGG
1970 1980 1990 2000 2010 2020 2030

Psp1406 I
|
BsrDI
|
GGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTG
CCTCGACTTACTTCGGTATGGTTTGCTGCTCGCACTGTGGTGCTACGGACATCGTTACCGTTGTTGCAAC
2040 2050 2060 2070 2080 2090 2100

FspI
|
AseI
|
CGCAAACCTATTAAGTGGCGAAGTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGG
GCGTTTGATAATTGACCGCTTGATGAATGAGATCGAAGGGCCGTTGTTAATTATCTGACCTACCTCCGCC
2110 2120 2130 2140 2150 2160 2170

Bgl I
|
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TATTTCAACGTCTCGGTGAAGACGCGAGCCGGAAGGCCGACCGACCAAATAACGACTATTTAGACCTCG
2180 2190 2200 2210 2220 2230 2240

BsaI
|
BpmI
|
BsrDI
|
CGGTGAGCGTGGGTCTCGCGGTATCATTTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTT
GCCACTCGCACCCAGAGCGCCATAGTAACGTCGTGACCCCGGTCTACCATTCCGGAGGGCATAGCATCAA
2250 2260 2270 2280 2290 2300 2310

AhdI
|
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TAGATGTGCTGCCCCCTCAGTCCGTTGATACCTACTTGCTTTATCTGTCTAGCGACTCTATCCACGGAGTG
2320 2330 2340 2350 2360 2370 2380

DraI
|
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ACTAATTCGTAACCATTTGACAGTCTGGTTCAAATGAGTATATATGAAATCTAACTAAATTTTGAAGTAA
2390 2400 2410 2420 2430 2440 2450

DraI
|
BspHI
|
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AATTAATTTTCTAGATCCACTTCTAGGAAAACTATTAGAGTACTGGTTTTAGGGAATTGCACTCAAA
2460 2470 2480 2490 2500 2510 2520

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2530 2540 2550 2560 2570 2580 2590

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2600 2610 2620 2630 2640 2650 2660

Eco57 I

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2670 2680 2690 2700 2710 2720 2730

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2740 2750 2760 2770 2780 2790 2800

AlwNI

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2810 2820 2830 2840 2850 2860 2870

ApaLI

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2880 2890 2900 2910 2920 2930 2940

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2950 2960 2970 2980 2990 3000 3010

BssSI

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3020 3030 3040 3050 3060 3070 3080

DrdI

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3090 3100 3110 3120 3130 3140 3150

BspLU11 I

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BsrBI

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3230 3240 3250 3260 3270 3280 3290

SapI
EaRI
|
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3300 3310 3320 3330 3340

Sites					Fragments				
	Number	Position	Length	Ends					
AatII GACGTC	1	1406	3337	1406-1405					
AccI GTIMKAC	3	254 738 893	2698 484 155	893-253 254-737 738-892					
Acil CIGC GCGG	34	19 29 85 188 1174 1213 1223 1249 1287 1300 1326 1335 1434 1481 1580 1689 1766 1810 1931 1977 2166 2259 2621 2630 2765 2875 2996 3015 3142 3170 3261 3282 3289 3332	986 362 191 135 127 121 121 110 109 103 99 99 91 91 77 56 47 46 44 43 39 38 28 26 26 24 21 19 13 10 10 9 9 7	188-1173 2259-2620 1977-2167 2630-2764 3015-3141 1810-1930 2875-2995 2765-2874 1580-1688 85-187 1335-1433 1481-1579 2168-2258 3170-3260 1689-1765 29-84 1434-1480 1931-1976 1766-1809 3289-3331 1174-1212 1249-1286 3142-3169 1300-1325 1223-1248 3332-18 3261-3281 2996-3014 1287-1299 19-28 1213-1222 1326-1334 2621-2629 3282-3288					
Afl II CITTAAG	1	1340	3337	1340-1339					
Afl III AICRYGT	2	611 3213	2602 735	611-3212 3213-610					
AgeI AICCGGT	1	277	3337	277-276					
AhdI GACNNININGTC	1	2325	3337	2325-2324					
AluI AGICT	16	56 120 215 237 776 994 1143 1162 1972 2035 2135 2656 2913 2959 3049 3275	810 539 521 257 226 218 149 118 100 95 90 64 63 46 22 19	1162-1971 237-775 2135-2655 2656-2912 3049-3274 776-993 994-1142 3275-55 2035-2134 120-214 2959-3048 56-119 1972-2034 2913-2958 215-236 1143-1161					
Alw26 I GTCTCNI INNNINGAGAC	4	1102 1144 1483 2259	2180 776 339 42	2259-1101 1483-2258 1144-1482 1102-1143					
AlwI GGATCNNNNI INNNINGATCC	14	260 273 522 804 817 911 1686 1690 2007 2470 2471 2567 2569 2655	942 775 463 317 282 249 96 94 86 13 13 4 2 1	2655-259 911-1685 2007-2469 1690-2006 522-803 273-521 2471-2566 817-910 2569-2654 260-272 804-816 1686-1689 2567-2568 2470-2470					
AlwNI CAGNNICTG	1	2804	3337	2804-2803					
ApaI GGGCGC	1	1091	3337	1091-1090					
ApaLI GITGCAC	3	1276 1653 2899	1714 1246 377	2899-1275 1653-2898 1276-1652					
ApoI RIAATTY	4	365 422 786 1008	2694 364 222 57	1008-364 422-785 786-1007 365-421					

	Sites		Fragments			Sites		Fragments	
	Number	Position	Length	Ends		Number	Position	Length	Ends
Asel	3	48	2043	107-2149			2207	258	1086-1343
ATITAAT		107	1235	2150-47					
		2150	59	48-106					
Asp718 I	1	274	3337	274-273		BpmI	2	336	1920 336-2255
GIGTACC						CTGGAGNNNNNNNNNN	256	1417	2256-335
						INNINNINNINNINNNC...			
AvaI	2	270	2897	710-269		BsaHI	2	1403	2955 1785-1402
CYCGRG		710	440	270-709		GRGCGC		1785	382 1403-1784
Avall	3	943	2097	2183-942		BsaI	1	2259	3337 2259-2258
GIGWCC		1961	1018	943-1960		GGTCTCNI			
		2183	222	1961-2182		INNINNNGAGACC			
BamHI	2	265	2793	809-264		BsaJI	5	137	2598 455-3052
GIGATCC		809	544	265-808		CICNGG		269	421 3053-136
								270	185 270-454
BanI	3	132	2098	274-2371				455	132 137-268
GIGYCC		274	1097	2372-131				3053	1 269-269
		2372	142	132-273					
BanII	2	996	3242	1091-995		BsaWI	5	277	1518 511-2028
GRGCGC		1091	95	996-1090		WCCGGW		511	831 2029-2859
								2029	607 3007-276
BbvI	11	65	887	65-951				2860	234 277-510
GCAGCNNNNNNNNNI		952	739	1152-1890				3007	147 2860-3006
INNINNINNINNINNNGCT		1152	419	2884-3302		Bsc4 I	8	34	1583 1151-2733
		1891	391	1891-2281		CCNNNNNNINGG		276	641 510-1150
		2282	303	2282-2584				510	279 2734-3012
		2585	206	2585-2790				1151	242 34-275
		2791	200	952-1151				2734	234 276-509
		2794	90	2794-2883				3013	174 3197-33
		2884	81	3321-64				3179	166 3013-3178
		3303	18	3303-3320				3197	18 3179-3196
		3321	3	2791-2793		BsiEI	6	1028	1062 3303-1027
Bcgl	1	1821	3337	1821-1820		CGFYCG		1081	923 1956-2878
GCANNNNNNNTCGNNNN...								1807	726 1081-1806
INNINNINNINNINCGANN...								1956	424 2879-3302
								2879	149 1807-1955
Bcgl	1	1787	3337	1787-1786				3303	53 1028-1080
CGANNNNNNNTGCNNNN...						BsiHKA I	5	996	1430 2903-995
INNINNINNINNINCGANN...						GWGCMC		1280	1161 1742-2902
								1657	377 1280-1656
BfaI	6	260	1059	1073-2131				1742	284 996-1279
CITAG		821	877	2720-259				2903	85 1657-1741
		1073	561	260-820		BsiWI	1	1082	3337 1082-1081
		2132	335	2132-2466		CIGTACG			
		2467	253	2467-2719					
		2720	252	821-1072		BsII	8	35	1583 1152-2734
Bgl I	3	1086	2216	2207-1085		CCNNNNNNINGG		277	641 511-1151
GCCNNNNINGGC		1344	863	1344-2206				511	279 2735-3013

Sites				Fragments	
	Number	Position	Length	Ends	
		1152	242	35-276	
		2735	234	277-510	
		3014	174	3198-34	
		3180	166	3014-3179	
		3198	18	3180-3197	
BsmBI	2	1102	3295	1144-1101	
CGTCTCNI		1144	42	1102-1143	
INNNNNGAGACG					
BsmFI	1	308	3337	308-307	
GGGACNNNNNNNNNNL...					
INNNNNNNNNNNNNNG...					
Bsp120 I	1	1087	3337	1087-1086	
GGGGOC					
Bsp1286 I	7	363	1161	1742-2902	
GDGCHC		996	797	2903-362	
		1091	633	363-995	
		1280	377	1280-1656	
		1657	189	1091-1279	
		1742	95	996-1090	
		2903	85	1657-1741	
BspEI	1	511	3337	511-510	
TCCGGA					
BspHI	3	1380	2224	2493-1379	
TICATGA		1485	1008	1485-2492	
		2493	105	1380-1484	
BspLU11 I	1	3213	3337	3213-3212	
AICATGT					
BspMI	1	240	3337	240-239	
ACCTGCNNNNNI					
INNNNNNNNNNGCAGGT ...					
BsrBI	3	188	1801	1483-3283	
GAGIOGG		1483	1295	188-1482	
CCGCTC		3284	241	3284-187	
BsrDI	2	2091	3163	2265-2090	
GCAATGNNI		2265	174	2091-2264	
ICATTGC					
BsrFI	3	277	1374	2240-276	
RICGGY		1023	1217	1023-2239	
		2240	746	277-1022	
BsrGI	1	565	3337	565-564	
TIGTACA					

Sites				Fragments	
	Number	Position	Length	Ends	
Bsrl	11	82	1238	442-1679	
ACTGGNI		319	608	2811-81	
CICAGT		442	406	2280-2685	
		1680	269	1850-2118	
		1850	237	82-318	
		2119	170	1680-1849	
		2162	123	319-441	
		2280	118	2162-2279	
		2686	112	2686-2797	
		2798	43	2119-2161	
		2811	13	2798-2810	
BssKI	11	137	875	270-1144	
ICNGG		269	696	2138-2833	
		270	607	1180-1786	
		1145	351	1787-2137	
		1180	288	3186-136	
		1787	218	2834-3051	
		2138	132	137-268	
		2834	121	3065-3185	
		3052	35	1145-1179	
		3065	13	3052-3064	
		3186	1	269-269	
BssSI	3	1349	1646	3040-1348	
CIACGAG		1656	1384	1656-3039	
CITCGTG		3040	307	1349-1655	
Bst1107 I	1	739	3337	739-738	
GTAITAC					
BstBI	1	911	3337	911-910	
TTICGAA					
BstF5 I	5	994	1984	2347-993	
GGATGNNI		1182	697	1182-1878	
ICATCC		1879	287	1879-2165	
		2166	188	994-1181	
		2347	181	2166-2346	
BstNI	4	139	2915	139-3053	
CCWGG		3054	288	3188-138	
		3067	121	3067-3187	
		3188	13	3054-3066	
BstUI	11	31	581	2589-3169	
CGIG		33	580	33-612	
		613	493	1766-2258	
		1102	489	613-1101	
		1104	332	1434-1765	
		1207	330	2259-2588	
		1434	227	1207-1433	

Sites		Fragments		Sites		Fragments			
Number	Position	Length	Ends	Number	Position	Length	Ends		
	1766	198	3170-30		1239	54	1089-1142		
	2259	103	1104-1206		1338	52	163-214		
	2589	2	31-32		1347	46	2913-2958		
	3170	2	1102-1103		1934	43	2685-2727		
BstYI RIGATCY	9	265	1030	2572-264		1972	41	2240-2280	
		809	768	1695-2462		2035	40	1195-1234	
		916	762	916-1677		2045	38	1934-1971	
		1678	544	265-808		2135	33	1162-1194	
		1695	107	809-915		2201	29	2656-2684	
		2463	86	2475-2560		2210	26	2214-2239	
		2475	17	1678-1694		2214	26	3147-3172	
		2561	12	2463-2474		2240	25	2883-2907	
	2572	11	2561-2571		2281	22	120-141		
Cac8 I GONINGC	12	58	975	1237-2211		2293	22	215-236	
		87	622	615-1236		2656	21	142-162	
		239	560	2603-3162		2685	19	3275-3293	
		243	391	2212-2602		2728	19	1143-1161	
		247	368	247-614		2739	18	3173-3190	
		615	152	87-238		2804	17	39-55	
		1237	109	3286-57		2883	12	1068-1079	
		2212	86	3200-3285		2908	12	2281-2292	
		2603	37	3163-3199		2913	11	3191-3201	
		3163	29	58-86		2959	11	2728-2738	
		3200	4	239-242		3049	10	2035-2044	
		3286	4	243-246		3147	9	1080-1088	
	Csp6 I GITAC	6	275	1769	1843-274		3173	9	2201-2209
			566	576	1267-1842		3191	9	1338-1346
		715	368	715-1082		3202	5	2908-2912	
		1083	291	275-565		3275	4	2210-2213	
		1267	184	1083-1266		3294	4	1235-1238	
		1843	149	566-714					
CviI I RGICY	48	39	587	1347-1933	DdeI CITNAG	7	1017	1415	2939-1016
		56	363	2293-2655			1270	540	1824-2363
		120	316	460-775			1398	426	1398-1823
		142	223	237-459			1824	409	2530-2938
		163	133	861-993			2364	253	1017-1269
		215	99	1239-1337		2530	166	2364-2529	
		237	98	3049-3146		2939	128	1270-1397	
		460	90	2959-3048	DpnI GATTC	17	267	955	2649-266
		776	90	2045-2134			516	726	918-1643
		861	85	776-860			811	341	2019-2359
		994	82	3294-38			918	295	516-810
		1068	79	2804-2882			1644	258	1697-1954
		1080	74	994-1067			1680	249	267-515
		1089	73	3202-3274			1697	107	811-917
	1143	68	2135-2200			1955	105	2360-2464	
	1162	65	2739-2803			2001	78	2477-2554	
	1195	64	56-119			2019	75	2574-2648	
	1235	63	1972-2034		2360	48	1955-2000		
					2465	38	1644-1679		
					2477	18	2001-2018		
					2555	17	1680-1696		

Sites				Fragments		Sites				Fragments			
Number		Position	Length	Ends		Number		Position	Length	Ends			
		2563	12	2465-2476				1141	328	2271-2598			
		2574	11	2563-2573				1250	245	1336-1580			
		2649	8	2555-2562				1336	229	1581-1809			
DraI TTTIAAA	4	679	1558	2458-678				1581	206	2599-2804			
		1747	1068	679-1746				1810	175	966-1140			
		2439	692	1747-2438				1905	155	3016-3170			
		2458	19	2439-2457				1932	143	2873-3015			
DrdI GACNNNNINNGTC	3	941	1915	1196-3110				2271	118	3171-3288			
		1196	1167	3111-940				2599	109	1141-1249			
		3111	255	941-1195				2805	95	1810-1904			
DsaI CICRYGG	1	455	3337	455-454				2808	86	1250-1335			
									2873	81	3310-53		
EaeI YGGGCCR	4	37	1442	1932-36				3016	65	2808-2872			
		458	854	1078-1931				3171	27	1905-1931			
		1078	620	458-1077				3289	18	3292-3309			
		1932	421	37-457				3292	3	2805-2807			
EagI CIGGCGG	1	1078	3337	1078-1077				3310	3	3289-3291			
EarI CTCTTCNI INNNGAAGAG	2	1526	1804	1526-3329				FokI					
		3330	1533	3330-1525				5	1001	1984	2354-1000		
Ecl136 II GAGICTC	1	994	3337	994-993				GGATGNNNNNNNNNNI ...1189					
									1189	697	1189-1885		
Eco57 I CTGAAGNNNNNNNNNN	3	639	1305	2671-638				INNNNNNNNNNNNNNC1886					
		1659	1020	639-1658						2173	188	1001-1188	
		2671	1012	1659-2670						2354	181	2173-2353	
EcoO109 I RGIGNOCY	2	1088	3080	1345-1087				FspI					
		1345	257	1088-1344						1	2102	3337	2102-2101
EcoRI GIAATTC	1	1008	3337	1008-1007				TGCIGCA					
EcoRII ICWGG	4	137	2915	137-3051				HaeII					
		3052	288	3186-136						3	6	1949	1024-2972
		3065	121	3065-3185						1024	1018	6-1023	
		3186	13	3052-3064						2973	370	2973-5	
FauI OCCGNNNNI NNNNNGCGGG	4	36	2157	1216-35				HaeIII					
		78	1128	78-1205						15	39	587	1347-1933
		1206	42	36-77						460	458	2281-2738	
		1216	10	1206-1215						861	434	2739-3172	
Fnu4HI GCNGC	19	54	912	54-965				GGCC					
		966	339	1932-2270						460	458	2281-2738	
											861	434	2739-3172

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		Number	Position	Length	Ends			Number	Position	Length	Ends	
HhaI GGGC	16	5	925	98-1022				1147	234	278-511		
		33	393	2196-2588				1181	190	2645-2834		
		98	337	1766-2102				1788	147	2861-3007		
		1023	332	1434-1765				2030	123	1024-1146		
		1104	270	3039-3308				2140	111	160-270		
		1207	227	1207-1433				2207	110	2030-2139		
		1434	174	2598-2871				2241	67	2140-2206		
		1766	109	2589-2697				2645	34	1147-1180		
		2103	103	1104-1206				2835	34	2207-2240		
		2196	100	2872-2971				2861	26	2835-2860		
		2589	93	2103-2195				3008	7	271-277		
		2698	81	1023-1103								
		2872	67	2972-3038				HphI	11	359	1214	2482-358
		2972	65	33-97				GGTGANNNNNNNNNI	398	476	647-1122	
		3039	33	3309-4				INNNNNNINTCACC	404	466	1132-1597	
		3309	28	5-32					647	416	1839-2254	
HinP1 I GGGC	16	3	925	96-1020				1123	243	404-646		
		31	393	2194-2586				1132	227	2255-2481		
		96	337	1764-2100				1598	206	1633-1838		
		1021	332	1432-1763				1633	39	359-397		
		1102	270	3037-3306				1839	35	1598-1632		
		1205	227	1205-1431				2255	9	1123-1131		
		1432	174	2696-2869				2482	6	398-403		
		1764	109	2587-2695								
		2101	103	1102-1204				KpnI	1	278	3337	278-277
		2194	100	2870-2969				GGTACIC				
		2587	93	2101-2193								
		2696	81	1021-1101				Maell	4	1403	2228	2512-1402
		2870	67	2970-3036				AICGT		1723	416	2096-2511
		2970	65	31-95						2096	373	1723-2095
		3037	33	3307-2						2512	320	1403-1722
		3307	28	3-30								
HincII GTYIRAC	2	255	2698	894-254								
		894	639	255-893								
HindIII AIAGCTT	1	235	3337	235-234								
HinfI GIANTC	7	42	1558	768-2325								
		256	517	2326-2842								
		768	512	256-767								
		2326	396	2843-3238								
		2843	214	42-255								
		3239	75	3239-3313								
		3314	65	3314-41								
HpaII CCGG	16	160	607	1181-1787								
		271	512	512-1023								
		278	489	3008-159								
		512	404	2241-2644								
		1024	242	1788-2029								
MbolI GAAGANNNNNNNNI INNNNNNINTCTTC	9	10	791	2556-9								
		314	755	1730-2484								
		695	728	815-1542								
		815	381	314-694								
		1543	304	10-313								
		1652	120	695-814								
		1730	109	1543-1651								
		2485	78	1652-1729								

Sites Number Position 2556 Fragments Length 71 Ends 2485-2555					Sites Number Position 2556 Fragments Length 71 Ends 2485-2555				
MfeI	1	849	3337	849-848	MwoI	12	11	863	1344-2206
CIAATTG					GCNNNNINNGC		95	789	238-1026
							139	572	2595-3166
							238	388	2207-2594
MluI	1	611	3337	611-610			1027	198	1086-1283
AICGCGT							1086	114	3167-3280
							1284	99	139-237
Mni I	13	32	762	376-1137			1344	84	11-94
CCTCNNNNNNNNI		256	594	1358-1951			2207	67	3281-10
NNNNNNINGAGG		376	400	2386-2785			2595	60	1284-1343
		1138	250	2786-3035			3167	59	1027-1085
		1358	224	32-255			3281	44	95-138
		1952	220	1138-1357					
		2158	209	3110-3318	NciI	7	271	875	272-1146
		2305	206	1952-2157	CCISGG		272	772	2836-270
		2386	147	2158-2304			1147	696	2140-2835
		2786	120	256-375			1182	607	1182-1788
		3036	81	2305-2385			1789	351	1789-2139
		3110	74	3036-3109			2140	35	1147-1181
		3319	50	3319-31			2836	1	271-271
MscI	1	460	3337	460-459	NcoI	1	455	3337	455-454
TGGICCA					CICATGG				
MseI	18	48	876	2509-47	NdeI	2	519	2573	1283-518
TITAA		107	563	678-1240	CAITATG		1283	764	519-1282
		354	372	1374-1745					
		420	365	1746-2110	NlaIII	17	226	720	2497-3216
		648	247	107-353	CATGI		245	491	2006-2496
		662	235	2150-2384			459	393	1489-1881
		678	228	420-647			533	390	553-942
		1241	100	1241-1340			553	346	3217-225
		1341	66	354-419			943	244	1140-1383
		1374	59	48-106			983	214	245-458
		1746	53	2385-2437			988	152	988-1139
		2111	52	2457-2508			1140	105	1384-1488
		2150	39	2111-2149			1384	78	1918-1995
		2385	33	1341-1373			1489	74	459-532
		2438	16	662-677			1882	40	943-982
		2452	14	648-661			1918	36	1882-1917
		2457	14	2438-2451			1996	20	533-552
		2509	5	2452-2456			2006	19	226-244
							2497	10	1996-2005
							3217	5	983-987
Msl I	3	1554	2819	2072-1553	NlaIV	12	134	772	2374-3145
CAYNNINNRTG		1913	359	1554-1912	GGNINCC		287	590	1438-2027
		2072	159	1913-2071			276	535	276-810
							811	349	1089-1437
MspA1 I	5	56	1157	56-1212			1089	286	3185-1331
CMGICKG		1213	941	1689-2629			1438	278	811-1088
		1689	518	2875-55			2028	211	2028-2238
		2630	476	1213-1688					
		2875	245	2630-2874					

Sites				Fragments		Sites				Fragments				
	Number	Position	Length	Ends		Number	Position	Length	Ends		Number	Position	Length	Ends
		2239	133	134-266			2017	75	2572-2646					
		2280	94	2280-2373			2358	46	1953-1998					
		2374	41	2239-2279			2463	36	1642-1677					
		3146	39	3146-3184			2475	18	1999-2016					
		3185	9	267-275			2553	17	1678-1694					
							2561	12	2463-2474					
							2572	11	2561-2571					
							2647	8	2553-2560					
NspI	3	245	2077	1140-3216	Sau96 I	9	860	1918	2279-859					
RCATGIY		1140	895	245-1139	GIGNOC		943	616	1345-1960					
		3217	365	3217-244			1087	257	1088-1344					
PleI	4	250	2084	250-2333			1088	222	1961-2182					
GAGTCNNNNI		2334	503	2334-2836			1345	144	943-1086					
NNNNINGACTC		2837	485	2837-3321			1961	83	860-942					
		3322	265	3322-249			2183	79	2200-2278					
Psp1406 I	2	1723	2964	2096-1722			2200	17	2183-2199					
AAICGTT		2096	373	1723-2095			2279	1	1087-1087					
PstI	1	251	3337	251-250	Scal	1	1844	3337	1844-1843					
CTGCAIG					AGTIACT									
PvuI	1	1956	3337	1956-1955	ScrFI	11	139	875	272-1146					
CGATICG					CCNGG		271	696	2140-2835					
PvuII	1	56	3337	56-55			272	607	1182-1788					
CAGICTG							1147	351	1789-2139					
RsaI	6	276	1769	1844-275			1182	288	3188-138					
GTIAC		567	576	1268-1843			1789	218	2836-3053					
		716	368	716-1083			2140	132	139-270					
		1084	291	276-566			2836	121	3067-3187					
		1268	184	1084-1267			3054	35	1147-1181					
		1844	149	567-715			3067	13	3054-3066					
							3188	1	271-271					
SacI	1	996	3337	996-995	SfaNI	9	385	1052	2064-3115					
GAGCTIC					GCATCNNNNNI		1167	782	385-1166					
Sal I	2	253	2698	892-252	NNNNNNNNNGATGC	1261	806	3116-384						
GITCGAC		892	639	253-891		1297	287	1337-1623						
SapI	1	3330	3337	3330-3329		1337	249	1624-1872						
GCTCTTCNI						1624	191	1873-2063						
NNNNINGAAGAGC						1873	94	1167-1260						
						2064	40	1297-1336						
						3116	36	1261-1296						
Sau3AI	17	265	955	2647-264	SfcI	4	247	1832	247-2078					
IGATC		514	726	916-1641	CITRYAG		2079	878	2079-2758					
		809	341	2017-2357			2757	836	2948-246					
		916	295	514-808			2948	191	2757-2947					
		1642	258	1695-1952										
		1678	249	265-513										
		1695	107	809-915										
		1953	105	2358-2462										
		1999	78	2475-2552										
					Sml	1	272	3337	272-271					

Sites					Fragments				
		Number	Position	Length	Ends			Number	Position
								Length	Ends
OOOOGGG								2063	211 1852-2062
SpeI	1	1072	3337	1072-1071		Tsp509 I	13	104	988 2453-103
AICTAGT						IAATT		179	884 1008-1891
								196	364 422-785
SphI	1	245	3337	245-244				327	306 2147-2452
GCATGIC								338	255 1892-2146
								365	159 849-1007
Sse8387 I	1	251	3337	251-250				422	131 196-326
CCTGCAIGG								786	75 104-178
								849	63 786-848
SspI	1	1520	3337	1520-1519				1008	57 365-421
AATIAAT								1892	27 338-364
								2147	17 179-195
StuI	1	1068	3337	1068-1067				2453	11 327-337
AGGICCT									
StyI	1	455	3337	455-454		TspRI	11	96	1524 382-1905
OCWWGG						CAGTGNIN		319	506 2818-3323
						CACTGNIN		382	347 1933-2279
								1906	271 2534-2804
Tail	4	1406	2228	2515-1405				1933	223 96-318
ACGTI		1726	416	2099-2514				2280	149 2385-2533
		2099	373	1726-2098				2385	109 3324-95
		2515	320	1406-1725				2534	105 2280-2384
								2805	63 319-381
								2818	27 1906-1932
								3324	13 2805-2817
TaqI	7	254	1444	1671-3114		XbaI	1	259	3337 259-258
TTCGA		657	760	911-1670		TICTAGA			
		711	476	3115-253					
		893	403	254-656		XhoI	1	710	3337 710-709
		911	182	711-892		CITCGAG			
		1671	54	657-710					
		3115	18	893-910		XmaI	1	270	3337 270-269
						OOOOGGG			
TII	3	42	2471	768-3238		Xmnl	1	1725	3337 1725-1724
GIAWTC		768	726	42-767		GAANNINNTTC			
		3239	140	3239-41					
TseI	11	53	912	53-964					
GCGGC		965	764	1140-1903					
		1140	419	2872-3290					
		1904	366	1904-2269					
		2270	328	2270-2597					
		2598	206	2598-2803					
		2804	175	965-1139					
		2807	81	3309-52					
		2872	65	2807-2871					
		3291	18	3291-3308					
		3309	3	2804-2806					
Tsp45 I	5	469	1743	2063-468					
IGTSAC		932	697	1155-1851					
		1155	463	469-931					
		1852	223	932-1154					

The following restriction enzymes do not cut pGFPuv:

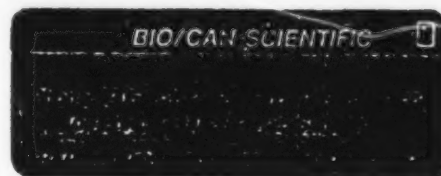
AscI, AvrII, BbeI, BbsI, Bcl I, Bgl II, BlpI, BsaAI, BsaBI, BseRI,

BsgI, BsmI, BssHII, BstEII, BstXI, Bsu36 I, ClaI, DraIII, Eco47 III,
EcoNI,

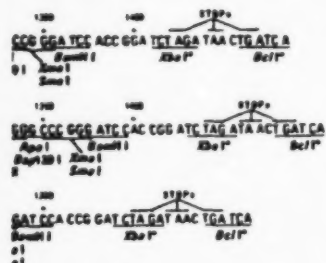
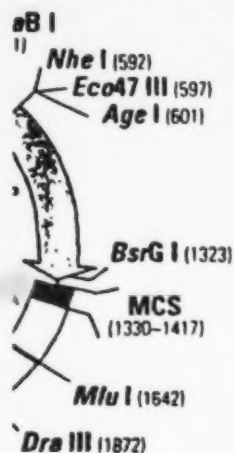
EcoRV, EheI, FseI, HpaI, Kasi, NaeI, NarI, NgoMI, NheI, NotI,

NruI, NsiI, PacI, PflMI, PmeI, Pml I, PpuI0 I, PpuMI, PshAI, RsrII,

SacII, SexAI, SgfI, SgrAI, SnaBI, SrfI, SwaI, TthIII I, XcmI.



continued



C2 and pEGFP-C3 are identical to
ame. Unique sites are in bold. The
CLONTECH. If you wish to digest
a *dam*⁻ host and make fresh DNA.
to generate fusions in pEGFP-C2
nces are deposited in GenBank
EGFP-C3 #U57607).

Appendix A. GFP Plasmid Maps & MCS continued

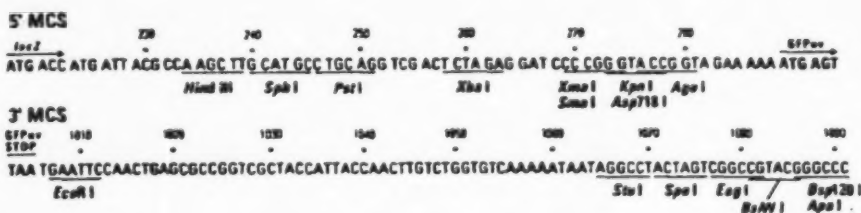
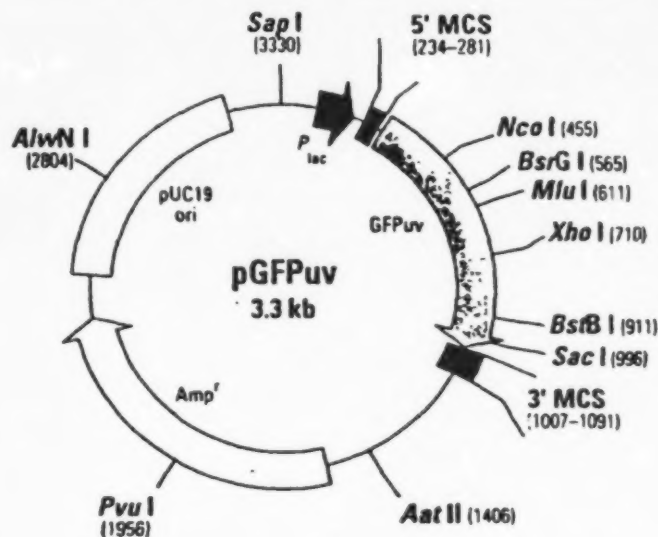


Figure 10. pGFPuv map and MCS. All sites shown are unique. The entire sequence is deposited in GenBank (Accession #U62636).

Appendix C. Primers For Cloning GFPuv (Clontech) Into pRSD12-8.

GFPuv 5' Not primer:

GFPuv 5' Not I primer was constructed from bases 289-307 of pGFPuv cloning vector (CLONTECH). The underlined bases are not complimentary to the vector and were added to engineer a Not I restriction site (in bold) for cloning scFv-GFPuv fusion. GFPuv 5' Not I primer is used in conjunction with GFPuv 3' Hind primer to generate a 738 bp PCR product (727 bp after Not I/Hind III digest).

5' G CGC GCG GCC GCG ATG AGT AAA GGA GAA GAA C 3'

GFPuv 3' Hind primer

GFPuv 3' Hind primer was constructed from bases 981-1009 of pGFPuv cloning vector (CLONTECH). The underlined bases are not complimentary to the vector and were added to engineer a Hind III restriction site (in bold) for cloning scFv-GFPuv fusion. GFPuv 3' Hind primer is used in conjunction with GFPuv 5' Not primer to generate a 738 bp PCR product (727 bp after Not I/Hind III digest).

5' TGG CAT GGA TGA GCT CTA CAA ATA ATG AAG **CTT** GTC T 3'
3' ACC GTA CCT ACT CGA GAT GTT TAT TAC **TTC** GAA CAG A 5'

5' A GAC AAG CTT CAT TAT TTG TAG AGC TCA TCC ATG CCA 3'

Inventory of Deliverables

Scientific Authority: E. Fulton, Defence Scientist
 Contract: W7702-6-R600/001/EDM
 Contract Title: GENERATION OF FLUORESCENT PROTEIN-SINGLE CHAIN
 ANTIBODY FUSION PRODUCTS AS BIO-DETECTION AND
 IDENTIFICATION REAGENTS

Items: Anti-Ovalbumin Mouse Monoclonal Hybridomas.
 Location: Liquid Nitrogen Freezer Facility, Tower B, Box B9 at DRES
 Date of Delivery: July 22, 1997

Primary Clones:

After a myeloma cell fusion cells were plated on multiwell plates. Cells are from a single well which are assay by ELISA to produce monoclonal antibodies to ovalbumin are collected as a 1st (primary) clone and named by location on plate and well (e.g. 7B11 indicates plate 7, well B11.)

<u>Sample</u>	<u>Source*</u>	<u>Date(s) Frozen</u>	<u>Quantity</u>
OVA7B11	FR24	5/13/97	1
OVA7C6	FR24	5/13/97	1
OVA7E10	FR24	5/13/97	1
OVA8G1	FR24	5/16/97	1
OVA2H7	FR24	5/16/97	1

Secondary Subclones:

Using the dilution method, individual cells from primary clones were plated into single wells. Resulting cloned cells which are assay by ELISA to produce monoclonal antibodies to ovalbumin are collected as a 2nd (secondary) subclones and named by location on plate and well (e.g. 7B11D5 indicates single cell from primary clone 7B11, grown in well D5)

<u>Sample</u>	<u>Source*</u>	<u>Date(s) Frozen</u>	<u>Quantity</u>
OVA1E11E9	FR24	5/22/97	1
OVA1E11E9	FR24	5/24/97	1
OVA1E11F6	FR24	5/22/97	1
OVA1E11F6	FR24	5/24/97	1
OVA1E11F11	FR24	5/22/97	1
OVA1E11F11	FR24	5/24/97	1
OVA8G1D5	FR24	5/22/97	1
OVA8G1D5	FR24	5/24/97	1
OVA8G1D7	FR24	5/22/97	1
OVA8G1D7	FR24	5/24/97	1
OVA8G1E8	FR24	5/22/97	1
OVA8G1E8	FR24	5/24/97	1

OVA12H6E6	FR24	5/24/97	1
OVA12H7E6	FR24	5/23/97	1
OVA12H6H8	FR24	5/24/97	1
OVA12H6E7	FR24	5/23/97	1

Tertiary Subclones:

Using the dilution method, individual cells from secondary subclones were plated into single wells. Resulting cloned cells which are assay by ELISA to produce monoclonal antibodies to ovalbumin are collected as a 3rd (tertiary) subclones and named by location on plate and well (e.g. 7B11D5D7 indicates single cell from secondary clone 7B11D5, grown in well D7).

<u>Sample</u>	<u>Source*</u>	<u>Date(s) Frozen</u>	<u>Quantity</u>
OVA1E11E9E5	FR24	5/28/97	1
OVA1E11F6E6	FR24	5/28/97	1
OVA1E11F11E4	FR24	5/28/97	1
OVA1E11E9E5	FRT25	5/29/97; 6.5/97	2
OVA1E11E9E11	FRT25	5/28/97; 5/29/97 6/8/97; 6/9/97	5
OVA1E11F6E5	FRT25	5/28/97; 5/29/97 6/5/97; 6/8/97	4
OVA1E11F6E6	FRT25	5/29/97; 6/9/97	2
OVA1E11F11E3	FRT25	5/28/97; 5/29/97 6/8/97; 6/9/97	4
OVA1E11F11E4	FRT25	5/29/97; 6/5/97 6/9/97	3
OVA8G1D5E2	FRT25/80	5/28/97; 5/29/97 6/4/97; 6/9/97	4
OVA8G1D5E7	FRT25/80	5/28/97; 5/29/97 6/4/97; 6/9/97	4
OVA8G1E8E1	FRT25/80	5/28/97 6/3/97; 6/5/97	3
OVA8G1E8F10	FRT25/80	5/28/97; 6/1/97; 6/3/97	4
OVA8G1D7E5	FRT25/80	5/28/97; 6/1/97; 6/3/97	4
OVA8G1D7E7	FRT25/80	5/28/97; 6/1/97; 6/3/97	4
OVA12H6E6F7	FRT25/80	6/1/97; 6/3/97 6/5/97; 6/9/97	5
OVA12H6H8F3	FRT25/80	6/1/97; 6/2/97 6/18/97	3

* Source indicates the what tissue culture container was used in the growth of cells for cell-freezing.

Abbreviations:

FR24 - from 24-well tissue culture plate.

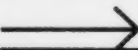
FRT25 -from tissue culture flask T25 (25 cm² flask)

FRT25/80 -from tissue culture flask T25 (25 cm² flask) or T80 (80 cm² flask).

Sample Array:

Since the positions in the box are not numbered a general scheme of how samples are put into the storage box as the user remove it directly, is shown below.

Samples are ordered from left to right as shown in list of deliverables above. A space separates the 3 general sample groups.



1 ^o Clones	space	2 ^o Subclones	
2 ^o Subclones		space	3 ^o Subclones
3 ^o Subclones			

Box-B1**Box B1-1. Bacterial glycerol stocks of general/expression *E. coli* strains.**

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
A1) TG1	11/18/96	Host <i>E. coli</i> for biopanning of rpCANTAB5E clones. F' episome selected on minimal media. Non-amber suppressor. Sub clone selected for higher transformation efficiency.
A2) BL21	3/5/98	Host <i>E. coli</i> strain for the expression of recombinant vectors. Sub clone selected for higher transformation efficiency.

Box B1-2. Bacterial glycerol stocks of pGFPuv (Clontech) vector.

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
C1) GFPuv	5/5/97	pGFPuv commercial vector from Clontech. Lac Z inducible promoter and ampicillin resistance gene. Transformed into TG1.
C2) GFPuv	5/5/97	pGFPuv commercial vector from Clontech. Lac Z inducible promoter and ampicillin resistance gene. Transformed into BL21.

Box B1-3. Bacterial glycerol stocks of Recombinant scFv-GFPuv fusion vectors (pRSD12guv in pRSET A).

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
E1) RSD12guv-1	5/14/97	Colony clone of PCR-amplified GFPuv cloned into RSD12-8 and transformed into TG1. pRSD12guv is an <i>E. coli</i> expression vector used to generate scFv-GFPuv fusion products. *Note: RSD12-8 is a colony clone of PCR-amplified D12 scFv cloned into pRSET A (a T7 based expression vector).
E2) RSD12guv-2	5/14/97	-as described above-
E3) RSD12guv-7	5/14/97	-as described above-
E4) RSD12guv-10	5/14/97	-as described above-
E5) RSD12guv-11	5/14/97	-as described above-

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
E6) RSD12guv-13	5/14/97	Colony clone of PCR-amplified GFPuv cloned into RSD12-8 and transformed into TG1. pRSD12guv is an <i>E. coli</i> expression vector used to generate scFv-GFPuv fusion products.
F1) RSD12guv-1	5/16/97	pRSD12guv-1 transformed into BL21.
F2) RSD12guv-2	6/02/97	pRSD12guv-2 transformed into BL21.
F3) RSD12guv-7	5/16/97	pRSD12guv-7 transformed into BL21.
F4) RSD12guv-10	5/16/97	pRSD12guv-10 transformed into BL21.
F5) RSD12guv-11	5/16/97	pRSD12guv-11 transformed into BL21.
F6) RSD12guv-13	6/02/97	pRSD12guv-13 transformed into BL21.

Box B1-4. Bacterial glycerol stocks of recombinant pCANTAB5E phagemid clones (mouse α -ovalbumin scFv clones).

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
G1) 12H6H8F3-2	8/06/97	Clone of 12H6H8F3 mouse α -ovalbumin scFv gene cloned into pCANTAB5E vector and transformed into TG1. LacZ inducible promoter and ampicillin resistance gene.
G2) 12H6H8F3-6	8/06/97	-as described above-
G3) 12H6H8F3-8	8/06/97	-as described above-
G4) 12H6H8F3-10	8/06/97	-as described above-
G5) 12H6H8F3-13	3/15/98	Clone of 12H6H8F3 mouse α -ovalbumin scFv gene cloned into pCANTAB5E vector and transformed into HB2151. LacZ inducible promoter and ampicillin resistance gene.

Box B1-5. Glycerol stock of Recombinant pCANTAB5E phagemid library of α -ovalbumin scFv clones.

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
G10) 12H6H8F3	3/05/98	Library of 12H6H8F3 mouse anti-ovalbumin scFv gene cloned into pCANTAB5E vector and transformed into TG1. LacZ inducible promoter and ampicillin resistance gene.

Box B1-6. Glycerol stock of Recombinant GFPuv-scFv fusion vectors (pGuvD12 in pGFPuv).

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
H1) GFPasc-1	3/15/98	SSAE linker cloned into the Sac I/Eco RI sites of pGFPuv. New scFv can be cloned at AscI/Spe sites. SSEA linker contains 6His and EK site for purification/detection. Transformed into TG1.
H2) GFPasc-2	3/15/98	-as described above-
H3) GFPasc-3	3/15/98	-as described above-

Box-P1**Recombinant Plasmid Stocks.**

These plasmid stocks are stored as ethanol-precipitated DNA (0.1 - 1 ug) and are to be used for transformation only in the event bacterial glycerol stocks are lost. DNA must be collected by centrifugation at 13K Xg /20 minutes/4°Cm washed with 70% ethanol, dried and resuspended in 10ul of sterile distilled water or TE buffer pH 8.0. Use 0.05 to .1ug of DNA/transformation assay.

Box P1-1. Plasmid DNA of pGFPuv (Clontech) vector.

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
A1) pGFPuv	3/19/98	pGFPuv commercial vector from Clontech. Lac Z inducible promoter and ampicillin resistance gene.

Box P1-2. Plasmid DNA of Recombinant scFv-GFPuv fusion vectors (pRSD12guv in pRSET A).

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
C1) pRSD12guv-1	3/19/98	Colony clone of PCR-amplified GFPuv cloned into RSD12-8 and transformed into TG1. pRSD12guv is an <i>E. coli</i> expression vector used to generate scFv-GFPuv fusion products. *Note: RSD12-8 is a colony clone of PCR-amplified D12 scFv cloned into pRSET A(a T7 based expression vector).
C2) pRSD12guv-2	3/19/98	-as described above-
C3) pRSD12guv-7	3/19/98	-as described above-
C4) pRSD12guv-10	3/19/98	-as described above-
C5) pRSD12guv-11	3/19/98	-as described above-
C6) pRSD12guv-13	3/19/98	-as described above-

Box P1-3. Recombinant pCANTAB5E phagemid DNA of α -ovalbumin scFv clones.

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
E1) p12H6H8F3-2	3/19/98	Plasmid DNA: Clone of 12H6H8F3 mouse anti-ovalbumin scFv gene cloned into pCANTAB5E vector. LacZ inducible promoter and ampicillin resistance gene.
E2) p12H6H8F3-6	3/19/98	-as described above-
E3) p12H6H8F3-8	3/19/98	-as described above-
E4) p12H6H8F3-10	3/19/98	-as described above-
E5) p12H6H8F3-13	3/19/98	-as described above-

Box P-4. Plasmid Library of Recombinant pCANTAB5E phagemid DNA of α -ovalbumin scFv clones.

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
G1) p12H6H8F3	03/25/98	Plasmid DNA: Library of 12H6H8F3 mouse anti-ovalbumin scFv gene cloned into pCANTAB5E vector. LacZ inducible promoter and ampicillin resistance gene.

Box P-5. Plasmid DNA of Recombinant GFPuv-scFv fusion vectors (pGuvD12 in pGFPuv).

<u>Vial Position Name</u>	<u>Date</u>	<u>Comments</u>
I1) pGFPasc-1	3/15/98	Plasmid DNA: SSAE linker cloned into the Sac I/Eco RI sites of pGFPuv. New scFv can be cloned at AscI/Spe sites. SSEA linker contains 6His and EK site for purification/detection.
I2) pGFPasc-2	3/15/98	-as described above-
I3) pGFPasc-3	3/15/98	-as described above-